

Do we still need astronauts?

Sending people into space for science is questionable and expensive. But a new proposed location for space telescopes, and the inevitable maintenance missions they will require, could provide a boost for the astronaut programme.

When humans first ventured into space in the 1960s, many people saw it as the opening of a new chapter of history, the beginning of a long, inevitable migration upwards and outwards. It hasn't worked out that way. In fact, it sometimes seems that the astronaut programme is merely playing out that initial burst of energy and is now in freefall, like a spent rocket arcing back to Earth.

One need look no further than the International Space Station, the centrepiece of NASA's human spaceflight programme, for evidence of inertia. Eighteen years and more than US\$20 billion into the project, it still has no agreed purpose. Economic justifications — better manufacturing processes, biotechnology breakthroughs, and so on — have been discredited. The most compelling remaining reason to send astronauts into space is to develop engineering skills and conduct biomedical studies that allow us to send more astronauts into space — a circular and ultimately hollow argument unless we really do intend to leave Earth's orbit again someday.

Do we? Mars has long been viewed as the next destination, but that planet is 200 times farther away than the Moon, and will be difficult and expensive for humans to reach. With no Apollo-style race to win, NASA appears to be in no hurry to send astronauts there, and has only robots in mind for Mars exploration in the foreseeable future.

Into this generally gloomy picture comes a new idea, and a new destination. A million miles from Earth is the L2 lagrangian (or libration) point, an ideal place to station telescopes as the combined gravitation of Earth and the Sun would fix them in place (see *News Feature*, page 666). At least eight new space observatories are planned to take up residence there by 2020. The question is whether astronauts will also make the trip, to repair and upgrade the telescopes.

For advocates of human spaceflight, L2 is an appealing short-term

destination, more adventurous than Earth's orbit but not as daunting as Mars. Like the missions to service the Hubble Space Telescope, it would stretch astronauts' skills, but not to breaking point. It seems possible, maybe even affordable.

But if we are to evaluate honestly whether astronauts are necessary for maintaining L2 telescopes, we have to ask whether robots could do the job just as well. To be fair, we should look well beyond today's capabilities. Outfitting astronauts for a million-mile journey, or even for the shorter trip to the vicinity of the Moon, as proposed by a team of NASA forward-thinkers, will cost billions and take at least a decade. If you gave the world's leading roboticists ten years and those same billions, what might they come up with? Only by running that experiment can we truly decide whether scientists need astronauts to build and service future space observatories.

If it turns out that they don't, we should look for other reasons to continue sending astronauts into space. Because people are risking their lives, and because human spaceflight is still fantastically expensive, one hopes for a deeper and more serious purpose than nostalgia (veteran astronaut John Glenn's 1995 shuttle flight was the most watched mission of the 1990s) or show business (reporters asked pop star and wannabe cosmonaut Lance Bass more questions than any of the professional astronauts at a recent NASA press conference).

For many people, space exploration touches a deep emotional nerve, and needs no further justification. Asked why he wanted to climb Everest, George Mallory famously replied, "Because it's there." Depending on your point of view, that rationale is either hopelessly glib, or the only truthful answer. And without a clear, practical job for astronauts to do in space — such as servicing telescopes — NASA will probably always be caught between those two viewpoints. ■

Towards a European Research Council

A meeting last week showed that Europe's science ministers should now focus on the bigger picture.

A bottom-up swell of lobbying from researchers in favour of the formation of a European Research Council (ERC) is about as likely as a thunderclap on the Moon. Only science-policy wonks are likely to have followed in detail the public discussion of the idea (see *Nature* **419**, 108–109; 2002 and **419**, 249–250; 2002). A correspondent on the topic (*Nature* **419**, 248; 2002) was sceptical about whether Europe is ready — such a funding agency would, it was argued, be bureaucratic and inevitably seek to take funds from existing bodies, which have themselves failed to foster international competitiveness.

Scepticism is understandable given the track record of some national agencies and of debates at the European level. On the other hand, centres such as the European laboratory for particle physics (CERN) and the European Molecular Biology Laboratory are comparatively efficient and have struck a balance between scientific independence and financial accountability. And they produce great science.

These examples may inspire, but a drive from the top down is now required if the idea of a pan-European funding agency, with fundamental research as its top priority and free to fund according to merit, is

to succeed. A meeting last week of stakeholders, including representatives of national funding agencies, was a good start. Organized in Copenhagen under the Danish presidency of the European Union, it achieved a strong degree of consensus that an ERC is indeed necessary, despite notable scepticism from the UK Medical Research Council and the CNRS, France's main basic research agency.

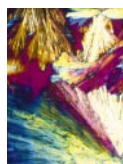
We can now hope for practical leadership, especially from Germany, the Netherlands and Scandinavia. The organization of heads of European national research councils (Eurohorcs) is likely to launch a scheme of young investigators' awards in which these and other countries will take a lead. And the European Commission's Sixth Framework Programme is set to boost the European Science Foundation's Eurocores collaboration scheme by 20 million euros (US\$19.7 million).

Given such positive signs, and the expressed aims of government heads to boost funds for European science, it would be appropriate for research ministers to establish a new goal: the formation by 2007 of an independent ERC, charged only with funding the best research, with an annual budget in excess of 5 billion euros. ■

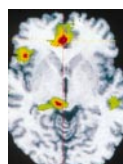




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Superweed study falters as seed firms deny access to transgene

Rex Dalton, San Diego

Two major seed companies this week stand accused of hindering attempts to assess whether genetically modified sunflowers can turn their wild counterparts into 'superweeds'.

A team led by Allison Snow, a plant ecologist at Ohio State University in Columbus, has uncovered preliminary evidence that a transgene that confers insect resistance can increase the number of seeds produced by wild sunflowers. This could allow the wild plants to proliferate as weeds.

But Pioneer Hi-Bred International of Des Moines, Iowa, and Indianapolis-based Dow AgroSciences have now blocked a follow-up study by refusing to allow the team access to either the transgene or the seeds from the earlier study.

Snow revealed the problem on 13 October during a lecture on transgene research at the Seventh International Symposium on the Biosafety of Genetically Modified Organisms in Beijing. "It is very frustrating," she said in an interview before the lecture. "We want to do good science. But this is keeping us from answering questions we want to ask."

The transgenic sunflowers contain a gene from the bacterium *Bacillus thuringiensis* (Bt), which allows them to produce a natural insecticide. Determining the impact of such transgenes in gene flow to wild plants is the subject of an intense scientific and political debate as nations consider what transgenic crops can be safely planted.

The results of Snow's initial study were revealed this August at the Ecological Society of America's annual meeting in Tucson, Arizona, and have been accepted for publication in *Ecological Applications*. They suggest that if the Bt transgene were to migrate into wild sunflowers, the plants' resistance to insects would increase the number of seeds produced. At a site in Nebraska, seed production in wild sunflowers with the transgene rose by 55%, although the results for plants at a site in Colorado were not statistically significant.

The refusal by Pioneer and Dow to pro-



Cold shoulder: Allison Snow has been left unable to follow up her experiments to test whether transgenes can cause wild sunflowers to proliferate as weeds.

vide transgenes for academic research is unusual, researchers in the field say. But *Nature* has identified at least one other recent case in which a plant geneticist at a leading US research university, who wanted to carry out an evolutionary study of Mexican maize, was denied access to transgenic material by two companies. That researcher does not want to be named.

Together with the US Department of Agriculture, Pioneer and Dow had funded Snow's study. But the seed firms say that they no longer want to continue the research because they do not plan to seek permission from the US government to sell transgenic sunflower seeds. Snow, however, wants to do further work, with purely academic funding, in an effort to confirm her initial findings.

Both seed companies confirm that they have refused to make the necessary materials available to Snow. "We would have to use time and talent to oversee the research," explains Doyle Karr, a spokesman for Pioneer. "We can't take the responsibility of putting the genes out if we aren't going to develop a product."

But critics of the company reject this. It

would be universities, not the suppliers of materials, that would be first in line for liability if any experiment went awry, they say. Mark Tepfer, a plant virologist at the French national agricultural agency's laboratory in Versailles, France, says that Snow's work is "one of the first really good experiments" to examine transgene flow to the wild. He called the companies' position "scandalous and indefensible".

Norman Ellstrand, a plant population geneticist at the University of California, Riverside, who often uses corporate transgenes for academic research without restrictions, says that the block imposed on Snow's team may be counterproductive for the biotechnology industry. It "only looks bad", he says, and could complicate future efforts to prove the biological safety of transgenic crops.

"There has to be a mechanism to conduct this type of research," says Gregory Jaffe, a biotechnology critic at the Center for Science in the Public Interest in Washington. "If the companies will not participate, then the government needs to step in and make sure researchers like Snow get the materials they need."

♦ www.worldbiosafety.net

Ecological footprint forecasts face sceptical challenge

Natasha McDowell, London

The concept of the 'ecological footprint' — the area of productive land and water that people need to support their consumption and to dispose of waste — is very much in vogue. London's footprint is 120 times as big as the land it covers, according to one well-publicized estimate; Hong Kong's may be 444 times so. And according to a report from the World Wide Fund for Nature (WWF), Earth's ecological footprint is in danger of growing larger than the entire planet.

But a report by the Danish Environmental Assessment Institute in Copenhagen — now run by environmental-policy specialist Bjorn Lomborg — has questioned the usefulness of the footprints as a research tool.

In his 2001 book *The Skeptical Environmentalist*, Lomborg used statistics to challenge widely held beliefs about the environment, and was criticized by many environmental scientists (see *Nature* 414, 149–150; 2001). His new report, *Assessing the Ecological Footprint*, released in August, claims that footprints are a weak analytical tool and are highly sensitive to changes in calculation method. "There is no 'correct' ecological footprint," Lomborg's team says.

In its *Living Planet Report 2002*, for example, the WWF claims that we will need between 1.8 and 2.2 Earth-sized planets to meet our needs by 2050. But Lomborg's team points out that this ignores the possible growth in the use of renewable resources. If, as many believe, the use of such resources increases, the 2050 footprint would not be so large. Lomborg also repeats a point made by some environmentalists — that ecological footprints provide snapshots of current resource use, but should not be used to generate predictions about the future. "It is a good effort, but the ecological footprint approach doesn't work right now," says Lomborg.

But many researchers say that the measure



Tread carefully: Bjorn Lomborg says that there are many ways to calculate ecological footprints.

is useful. Advocates argue that footprints help to summarize information, and are no more of a gimmick than such well-accepted yardsticks as gross domestic product. "No single indicator is a good measure, as every time you collapse lots of diverse information you lose something," says Simon Levin, an ecologist at Princeton University in New Jersey. "But they are useful, in the same way as indicators helping people judge the state of the economy are."

Robert Watson, chief scientist at the World Bank, admits that footprints cannot be used as predictive tools, but says that they can help to weigh up different options, provided that the underlying assumptions are made clear. "It is perfectly valid to ask what the result of continuing to produce energy using predominantly fossil fuels will be," Watson says. "But we must then ask what the footprint will be if we have a different way of producing energy." If we don't make the shift to renewable energy sources, for example, the WWF's model may end up being accurate, he says.

Watson is also co-chair of the Millennium Ecosystem Assessment, a four-year effort to survey the health of the world's ecosystems (see *Nature* 417, 112–113; 2002). Footprints may yet be included as part of the assessment. "We will examine the approach to see how useful it is and to what degree it is a quantitative concept," Watson says.

Researchers who have criticized the use of ecological footprints say that Lomborg has exaggerated their views. Ian Moffat, an ecologist at the University of Stirling, UK, is quoted in the institute's report as arguing that the concept is "nothing more than an important attention-grabbing device". But Moffat claims that Lomborg took his remarks out of context and that he regards footprints as valuable in showing the scale of ecological problems. ■

Greenhouse gas that preserves ozone puts protocols at odds

Quirin Schiermeier, Munich

A head-on collision between efforts to combat climate change and those aimed at protecting the ozone layer is on the cards when delegates from 146 nations meet in New Delhi next week to discuss greenhouse-gas emissions.

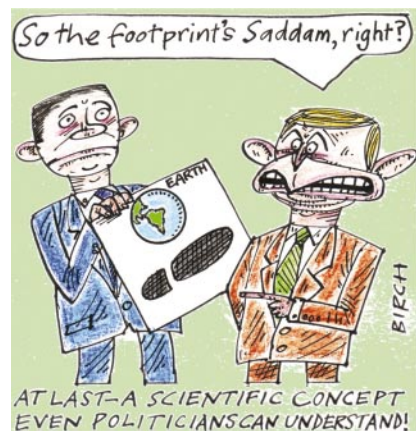
At issue are hydrofluorocarbons (HFCs), introduced to replace chlorofluorocarbons (CFCs) under the 1987 Montreal Protocol, the international agreement on protecting the ozone layer. But the Kyoto Protocol, which aims to limit greenhouse-gas emissions, warns against using HFCs, as they have a global-warming potential 1,300 times that of carbon dioxide.

HFCs, which are used in applications such as air-conditioning systems, currently account for less than 2% of global greenhouse-gas emissions. But according to a study published in June by the Brussels-based Climate Action Network Europe (CAN Europe), growing markets for HFCs in developing countries are likely to lead to a tenfold increase in HFC emissions by 2050. "There is a glaring contradiction between the two initiatives," says Jason Anderson, an energy-technology expert at CAN Europe.

At the New Delhi meeting, which starts on 23 October and is intended to discuss the implementation of the Kyoto Protocol, delegates may ask technical advisers to the two treaties to carry out a joint study of other CFC replacements. Hydrocarbon alternatives, such as isobutane and pentane, are already used in Europe and by a growing number of manufacturers in Japan and China.

But US industry uses HFCs — which, unlike the hydrocarbon alternatives, are not flammable. Developing countries also pose problems. They receive annual subsidies worth US\$150 million under the Montreal Protocol to convert their industries to ozone-friendly technologies. In most cases, this is used for the relatively cheap switch to HFCs. Opting for a technology tends to cement its use for many years, says Stephan Sicars, a member of the Technology and Economic Assessment Panel of the Montreal Protocol.

The CAN study criticizes this switch. "Using subsidies to switch to HFCs is a lost opportunity," says Anderson. "It also makes developing countries vulnerable to future greenhouse-gas-reduction requirements." Like many experts, he believes the lack of explicit links between the two protocols lies at the heart of the problem. "The technical study can help open people's eyes," he says. "But without a firm policy commitment, it won't solve the principal dilemma." ■



Japan's innovators take patent deals to court

David Cyranoski, Tokyo

Not long ago, the typical response of a Japanese researcher who thought that their employer had stolen their ideas would have been a brief shrug of resignation. Often, they would have been content to entrust their fate to the company in exchange for gradual promotion over a lifetime of employment.

But times are changing, and these days more and more peeved innovators are opting for a different response — they're calling in a lawyer.

Industrial researchers in Japan are awarded patents as individuals, but they customarily sign these rights over to their employers. Patent law holds that researchers must be awarded "reasonable compensation" for patents that become lucrative.

The law doesn't say what "reasonable" means. But after last year's widely publicized lawsuit filed by Shuji Nakamura, inventor of the blue light-emitting diode (see *Nature* 412, 844; 2001), more researchers are going to the courts to find out.

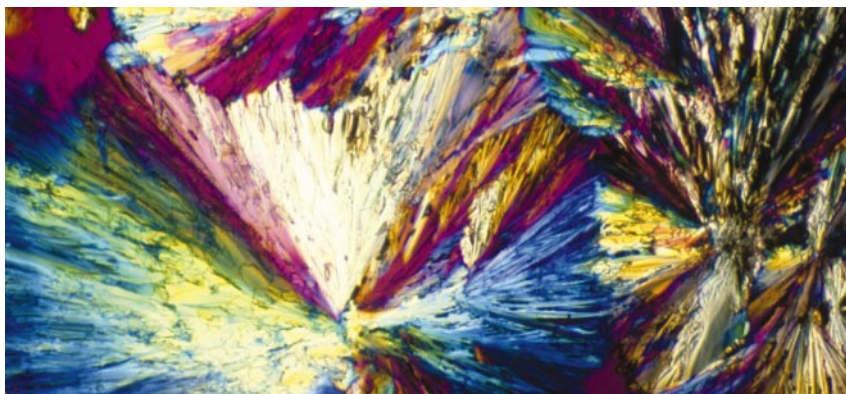
In a lawsuit filed last month in the Tokyo district court, for example, Masayoshi Naruse says that the ¥10 million (US\$80,000) he received in 2001 for his work on the artificial sweetener aspartame does not match up to the ¥23 billion that his former employer, Tokyo-based Ajinomoto, reportedly made from licensing the product in the United States between 1982 and 2000. His suit demands half of the profits made on the sweetener — with ¥2 billion as a first instalment.

In another lawsuit filed in the same court on 2 October, Hiroshi Ogawa took issue with the ¥100,000 he received for his patent on processing the vitamin-like substance inositol. Ogawa says that the patent, on a procedure to extract inositol from corn, has earned close to ¥2 billion for his former employer Shikishima Starch and its parent company Showa Sangyo of Tokyo. Ogawa is claiming 80% of these profits on the basis that the company actively sought to sideline his work. "They resist anything new," he says.

Ogawa and Naruse are also claiming that they never legally transferred the patents to their employers and that control of the patents should revert to the discoverers.

In each case, the defending companies claim that they received the patents legally, and that the compensation given was fair. Ajinomoto says that Naruse's award was calculated as a percentage of profits according to a company compensation formula established in 1999 — and that his patent is just one of many related to the discovery and production of aspartame.

The cases reflect a new assertiveness on the part of Japan's industrial researchers.



Sweet crystals of success: but aspartame's inventor claims he was short-changed by his employers.

"The problem is that nobody has used this patent law to get reasonable compensation from a company until now, either because the researchers were very loyal to their companies or because they didn't understand patent law," says Nakamura, now at the University of California, Santa Barbara.

As researchers become more aware of the profits to be made and less reliant on lifetime employment, Japan's employers are changing their approach. In the past few years, many companies, including Honda and Toyota, have revamped their reward systems to give researchers greater motivation. ■

Words but no cash for US agencies

Geoff Brumfiel, Washington

The US National Science Foundation (NSF) got some good news last week, when a House committee proposed a 13% increase in its funding for the 2003 fiscal year, which began on 1 October. But, for the NSF, and every other agency of the federal government, the big question is when a gridlocked Congress will get around to passing any budget at all.

As lawmakers return to their home states for this November's elections, the federal government faces its biggest budget log-jam in years. None of the 13 appropriations bills that fund the government has been agreed by Congress and signed by the president. All agencies are instead getting money at 2002 levels under a 'continuing resolution'. So, for agencies such as the National Institutes of Health (NIH), which had been anticipating a huge \$3.7-billion funding increase, plans for spending the extra money are on hold.

The budget is stalled because neither the Democrat-controlled Senate nor the Republican-led House can agree spending levels that fall within the overall budget proposed by President Bush back in February.

At the head of the snarl-up in the House is the labour, health and education bill, which funds the NIH: the House leadership wants it to pass at the level suggested by President Bush, but Democrats and some moderate Republicans say it doesn't contain

enough money to meet their various spending priorities.

Elias Zerhouni, director of the NIH, told Congress last week that a long-term continuing resolution will delay construction of a new clinical centre on the NIH campus and stall its \$1.5-billion plan for bioterrorism research. Also, says Pat White of the Federation of American Societies for Experimental Biology, it could cut by a third the number of new grants that the NIH can make in the coming year's first round of awards, due in December.

Continuing resolutions will nonetheless probably extend at least until after the elections. The old Congress may then seek to resolve the budget in a 'lame duck' session before the new Congress arrives in January — but the progress of such a session will hinge on the election outcome, and it is quite possible that the flat funding could continue into January or beyond. "The wheels have come off the budget process," White says.

So the proposed NSF increase — a large advance on the 5% hike proposed by President George Bush in February (see *Nature* 415, 564; 2002) — leaves science lobbyists only moderately thrilled. "All of us are pleased with the House's recommendation," says Samuel Rankin, chair of the Coalition for National Science Funding, which advocates doubling NSF funding over five years. "But it won't mean anything if there isn't a budget." ■

Top London colleges consider merger to form research giant

David Adam, London

In a move that has sent shock waves through both colleges, London's two largest research institutions have revealed that they are considering plans to merge. University College London (UCL) and Imperial College say that they are discussing the move in a bid to compete more effectively with top US institutions.

If the merger happens, it will create a university with 28,000 students and almost £400 million (US\$620 million) in yearly research funds — far more than Oxford or Cambridge, which will each spend about £200 million on research this year. A decision to merge could be made as early as this December, with the new institution taking its first steps by this time next year.

The possible merger is a “once-in-a-lifetime opportunity”, says Derek Roberts, provost of UCL. “We’re talking about creating the world’s leading university, full stop. We see this as an institution not for the next two or three years but for two or three centuries.”

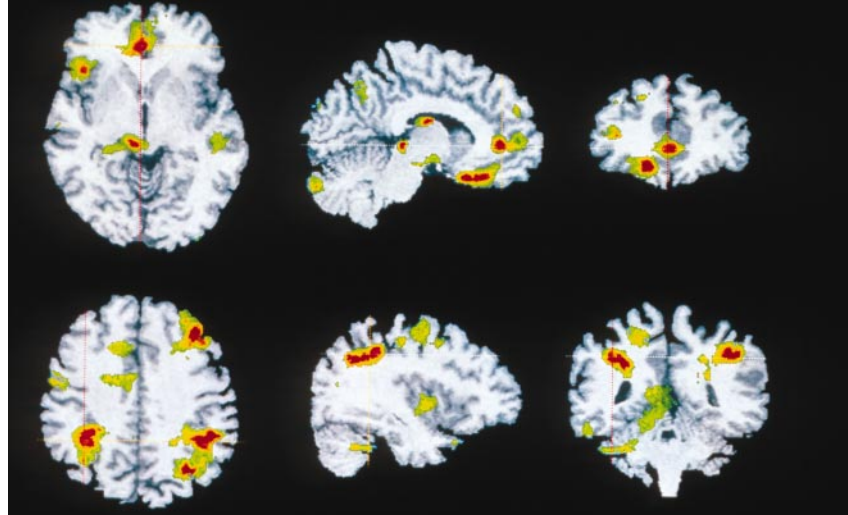
Richard Sykes, rector of Imperial College, says that both colleges are embarking on a consultation exercise “to ensure that if the merger does go ahead we carry most people with us”. If it does happen, the new body is likely to leave the loosely federated University of London.

Roberts and Sykes both have industrial backgrounds, and it isn't clear if academics at the universities will share their enthusiasm for a larger institution that would combine Imperial's strengths in engineering and the physical sciences with UCL's broader tradition in science, medicine and the humanities.

“Everyone's a bit shell-shocked round here and its causing a lot of concern,” says one physicist at UCL, who did not want to be identified. “To get any benefit from a merger they'll have to re-site a lot of people and while that's sorted out, there will be enormous disruption.”

A joint working group will report to the respective universities' ruling bodies at the end of the year — although a formal merger would require an act of parliament. One issue that will not complicate any potential combination is leadership. Sykes would head it, as Roberts is only in charge of UCL temporarily (see *Nature* 418, 576; 2002).

The British government is preparing a discussion paper on higher education that is widely expected to intensify competition for cash and students between the country's 114 universities. ■



Knowledge of the brain areas active in obsessive disorders makes them ideal for deep brain stimulation.

Brain implants show promise against obsessive disorder

Alison Abbott, Rome

A controversial experimental technique for treating certain psychiatric disorders is producing encouraging results, according to the neurologists who are testing it.

The technique, known as deep brain stimulation, uses an electric current from implanted electrodes to modulate brain function and has already raised ethical concerns (see *Nature* 417, 677; 2002).

But at the The Human Brain, an international conference held in Rome earlier this month, researchers said that the technique is showing promise. As a result, they pledged to set up an international collaboration to speed its development.

At the meeting, neurosurgeon Volker Sturm of the University of Cologne presented data on 3 of the first 20 or so patients to have the therapy for obsessive-compulsive disorder. All showed improvements in their symptoms, he said. One patient, for example, who had been unable to look after her young son because of her compulsion to check repeatedly if he had been suffocated by his pillows at night, showed a marked reduction in her compulsion a few weeks after the treatment, Sturm said.

Bart Nuttin, of the Catholic University of Leuven in Belgium, published the first pilot study of four patients using this technique (B. Nuttin *et al.* *Lancet* 354, 1526; 1999), which has now been replicated at three centres in the United States. These centres plan

to join several others in Europe to cooperate on optimizing the technique.

Ethical guidelines for the use of deep brain stimulation to treat psychiatric illness were published two months ago (B. Nuttin *et al.* *Neurosurgery* 51, 519; 2002). The technique has already been used to help hundreds of people with Parkinson's disease to move more normally, says Sturm, who has operated on over 200 Parkinson's patients.

The neural pathways that underpin obsessive-compulsive disorder are better understood than those of most psychiatric disorders, which means that very precise anatomical structures in the brain can be targeted by neurosurgeons. Some 15% of people with the disorder commit suicide, although prospects for patients who are resistant to drug or behavioural therapy have been improved by lesion of brain structures known as internal capsules. But such surgery is irreversible. “Deep brain stimulation is theoretically preferable to lesioning,” says Nuttin. “An electrode can be switched off or removed.”

The international collaboration will seek to determine the most effective location for the electrodes and the optimal level of current. Most of the operations performed so far have placed electrodes in the anterior limbs of the internal capsules. Sturm places his electrodes in a nearby structure, the shell of the nucleus accumbens. “We have been able to use a much lower level of current than studies using implants in the internal capsules,” he says.

Neurologists do not understand how the technique brings obsessive-compulsive disorder under control. Suppression of obsessive symptoms can require weeks of stimulation, although a patient's mood can be altered within seconds. In contrast, the clinical improvement derived from the technique in Parkinson's patients is immediate. ■



Volker Sturm: electrode therapy can help.

Structured approach bags chemistry prize

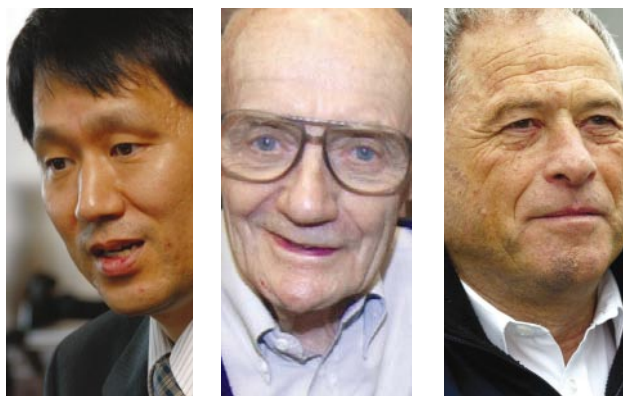
David Adam, London

Discerning the shape and structure of biomolecules is a sizeable problem — huge, complicated structures such as proteins are among the toughest molecules to analyse. Three researchers who developed key tools to study these giants have been rewarded with the Nobel Prize in Chemistry.

Half of the prize goes to Kurt Wüthrich of the Swiss Federal Institute of Technology in Zürich and the Scripps Research Institute in California, for finding ways to determine the three-dimensional structures of large biological molecules using nuclear magnetic resonance (NMR) spectroscopy.

John Fenn of the Virginia Commonwealth University in Richmond and Koichi Tanaka of the Shimadzu Corporation in Kyoto share the other half for inventing techniques to identify and analyse proteins and other large structures using mass spectrometry. At 43, Tanaka is the youngest chemistry laureate since 1967, and the second Japanese scientist to receive a Nobel this year, following physics winner Masatoshi Koshihara.

"The possibility of analysing proteins in detail has led to increased understanding of the processes of life," says the Nobel Foundation. "Researchers can now rapidly and simply reveal what different proteins a sample contains and also determine what protein molecules look like in solution."



Broad spectrum: techniques devised by (from left) Koichi Tanaka, John Fenn and Kurt Wüthrich have helped to reveal the secrets of protein structure.

Chemists have used NMR and mass spectrometry for decades to study small molecules. But the large size and complex structure of proteins posed problems for biologists wanting to do the same.

NMR analyses the way a molecule's atoms absorb radio waves in a powerful magnetic field. Proteins can contain thousands of atoms, so they give highly confusing NMR spectra. But in the 1980s, Wüthrich showed that NMR is possible for proteins. He invented 'sequential assignment' in which he determined the distance between any two hydrogen atoms in the molecule. He could

then pair each peak of radio absorption with a hydrogen nucleus in the protein. This allowed the structure of proteins to be determined in the form in which they exist in the body — in solution — rather than as crystals.

Mass spectrometry is a highly sensitive analytical tool that separates molecules according to their size. Fenn and Tanaka found ways of turning proteins into a charged

vapour, to be accelerated by an electric field and detected in a mass spectrometer.

Tanaka's technique — soft laser desorption — uses a laser pulse to blast material from solid or viscous biological samples. Fenn developed a different approach, electrospray ionization, which creates a fine spray from a protein solution using an electric field.

Fenn has "been in a total state of shock" since being given the news in a dawn phone call on 9 October. "It's like being struck by lightning," he says. "You know it happens to some people but the odds are so great you never believe it will happen to you." ■

Economists honoured for experimental angle

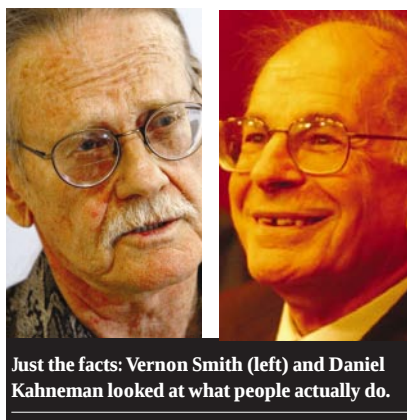
Rory Howlett, London

Two researchers who put practical ideas to work in a theory-dominated discipline have netted this year's Nobel prize in economics.

Daniel Kahneman of Princeton University in New Jersey integrated psychology into economics, showing how human judgement and decision-making help to shape economic processes. He shares the prize with Vernon Smith of George Mason University in Virginia, who pioneered the use of real experimental data as a check on economic theory.

"Economics textbooks are full of theory, but have no data or experimental results," explains Herbert Gintis of the University of Massachusetts in Amherst. "We need to understand how people actually behave and how this affects economic institutions."

Classical economic theory rests on the idea that people will behave rationally, in their own best interests. Kahneman was among the first to show that people's economic choices are not always rational, however, and can be strongly influenced by their perceptions and emotions. He found



Just the facts: Vernon Smith (left) and Daniel Kahneman looked at what people actually do.

that people often display biases in their economic behaviour that lead to patterns of decision-making that are at odds with what traditional theory predicts.

Having vanquished orthodoxy, Kahneman and the late Amos Tversky proposed a new framework in a classic paper, "Prospect theory: An analysis of decision under risk" (D. Kahneman & A. Tversky *Econometrica* 47, 263–291; 1979). Unlike the

ideas that it challenges, prospect theory uses empirical observations to generate specific hypotheses that can be tested experimentally.

Smith, meanwhile, has made extensive use of carefully controlled experiments to study how markets behave.

According to economist Samuel Bowles, at the Santa Fe Institute in New Mexico, Smith showed that the standard economic model of how markets work is correct, even as Kahneman proved that the model of rational economic decision-making is wrong. This apparent paradox makes sense, Bowles says, because market models "do not require any particular kind of rationality or high-level cognitive capacity" on the part of participants. He concludes that "the main difference between biological and economic competition — namely, the rationality of the human agents — may be overrated".

Gintis says that Kahneman and Smith's ideas were slow to catch on in academia. But Harvard University, Massachusetts Institute of Technology and the University of Chicago have each now appointed senior faculty in the subdisciplines the duo pioneered. ■

FDA gives green light to immunodeficiency gene-therapy trials

Washington US trials of gene therapy for a rare immune-system disease are to continue, the Food and Drug Administration (FDA) said on 10 October. A similar study in France was halted in September after a child developed a leukaemia-like illness (see *Nature* **419**, 545–546; 2002).

An FDA advisory committee said the gene therapy was probably to blame for the French incident, but that the US trials should go ahead anyway. “One adverse event is not enough to advise the FDA to

put all these trials on hold,” said the committee’s chair, xenotransplantation researcher Daniel Salomon of the Scripps Research Institute in La Jolla, California.

The study involves patients with severe combined immunodeficiency disease, which disrupts the early development of the immune system. The committee also said that patients in about 150 other similar studies in the United States should be told of the adverse developments in the French trial.

In addition, the FDA last week approved the first gene-therapy trial to treat Parkinson’s disease. This will use a virus to insert a gene for a neurotransmitter into the brains of patients with Parkinson’s, an approach that animal models suggest may be effective (J. Luo *et al. Science* **298**, 425–429; 2002).

Siberian fireball sightings spur meteorite hunt

Moscow Russian space scientists are investigating reports that a large meteorite struck a remote area of Siberia last month.

Witnesses reported seeing a fireball fall to Earth near Bodaibo, in the Irkutsk region, at 1.50am on 25 September. Researchers from the Institute of Solar–Terrestrial Physics in Irkutsk are believed to be heading to the region to discover whether eyewitnesses can

help them find the impact zone.

But Mikhail Nazarov, a meteorite researcher at the Vernadsky Institute of Geochemistry and Analytical Chemistry in Moscow, cautions that the event may not be as significant as first appears. “A fireball is very impressive and eyewitnesses who have never seen one before will exaggerate its effect,” he says.

Global science council names new head

New Delhi Indian chemist Goverdhan Mehta has been named president-elect of the International Council for Science (ICSU), the Paris-based organization that aims to bring together scientific researchers across the world.

Mehta has been director of the Indian Institute of Science in Bangalore since 1998 and specializes in organic chemistry. His term as president-elect will begin next March, and he will take over as president from Jane Lubchenco, a marine ecologist from Oregon State University in Corvallis, in November 2004.

ICSU, which comprises 98 national science academies and research councils, and 26 international scientific unions, has been particularly active in addressing issues concerning sustainable development and



Bubble baby: isolation used to be the only way to keep severely immunodeficient children alive.

in promoting science initiatives in the developing world. At this summer's World Summit on Sustainable Development in Johannesburg, Lubchenco and colleagues helped to run meetings on science and sustainable development, whose results will be released early next year (see *Nature* **418**, 812–814; 2002).

South African countries pull together on transgenic food

London Southern African countries have set up their own advisory committee to investigate the potential risks associated with genetically modified crops.

The move is a response to the refusal of some of the region's drought-stricken countries to accept US food aid because of the likelihood that it contains transgenic seeds (see *Nature* **418**, 571–572; 2002). The committee, which was announced at the Southern African Development Community summit in Luanda, Angola, at the start of this month, will develop guidelines on issues such as food safety and consumer concerns.

Delegates also agreed that the 14 countries of the community can decide individually whether to accept genetically modified food, but that any transgenic grain must be milled before distribution. They said that awareness



Aid alarm: fears over transgenic maize delayed the distribution of US food supplies in Africa.

campaigns should also be undertaken to ensure that the grain is not planted.

Search for bountiful plants receives boost

San Diego The sometimes controversial field of bioprospecting is to benefit from a fresh round of funding from the US National Institutes of Health (NIH).

A call for proposals for the International Cooperative Biodiversity Groups programme, which aims both to identify plants possessing useful pharmaceutical ingredients and to

encourage sustainable development in developing nations, was announced earlier this month. The scheme already has five projects active in Asia, Central and South America and Africa. Researchers withdrew from a sixth study in Mexico last year, in part because environmentalists had accused them of exploiting indigenous resources for commercial ends (see *Nature* **414**, 685; 2001).

Science and engineering not quite Mr Right

London The message is familiar; the messenger is not. November's UK edition of the women's magazine *Good Housekeeping* carries a survey of women's attitudes to science which it says reveals "a criminal waste of female talent".

UK government figures quoted show that about 50,000 female science, engineering and technology graduates are not working as such at any given time. A quarter of the women interviewed admitted telling their daughters "never mind, I was never any good at science or maths at school either".

"Unless we change the culture that says science is unsexy for women we are going to continue to deprive scientific research of some of the best brains in the country," says Lindsay Nicholson, *Good Housekeeping's* editor-in-chief.



How many more fish in the sea?

Commercial fisheries worldwide are being driven to collapse. Quirin Schiermeier wonders why fisheries scientists are failing to halt this pillage, and asks what hope is there for the future sustainability of fish stocks.

For centuries, the Canadian Grand Banks, off the coast of Newfoundland, were prime fishing grounds. The region's abundance of Atlantic cod (*Gadus morhua*) supported entire communities. At its height, in 1968, the industry employed 40,000 people and landed more than 800,000 tonnes of the fish.

But the factory trawlers that subsequently moved onto the banks exacted a dreadful toll. Stocks collapsed, and in 1992, Canada's Department of Fisheries and Oceans belatedly closed the fishery. Thousands of fishermen and workers in the fish-processing industry lost their jobs; others redirected their efforts to crabs and shrimp. A decade later, the Grand Banks' cod stocks show little sign of recovery.

The Grand Banks disaster shows just how badly fisheries policies can go wrong. And unfortunately, the same mistakes are being

made across the world's oceans. In many cases, scientists are warning that populations are being overexploited. But all too often, their advice of setting lower catch quotas, reducing the size of fishing fleets and using less harmful fishing gear is ignored or watered down. When push comes to shove, it seems that short-term economic interests steamroller scientific arguments.

In part, fisheries scientists are cursed by the uncertainties that swathe their work. At best, their models of the dynamics of fish populations produce imprecise estimates of the maximum catches that can be taken without driving a stock to extinction. Here, it's easy for those with vested interests to ignore unpalatable messages and to argue that larger catches might be sustainable. At worst, the models can incorporate misleading data that simply give the wrong answer, causing scientists to help speed fisheries towards collapse. In the case of the Grand Banks, both scenarios came into play.

Given such failures, some conservation biologists are now arguing that fisheries scientists must abandon their focus on individual stocks and adopt a whole-ecosystem perspective. But whatever methods are used to determine the advice given to policy-makers, scientists need to find ways to involve fishermen in their work, to break down the 'us-and-them' interaction that helps to foster the current gulf between science and policy. "It is utterly important to get fishermen's legitimate inter-

ests involved," says John Pope, an independent consultant and former chief scientist at the Lowestoft Laboratory in East Anglia, part of Britain's Centre for Environment, Fisheries and Aquaculture Science.

Scale of the problem

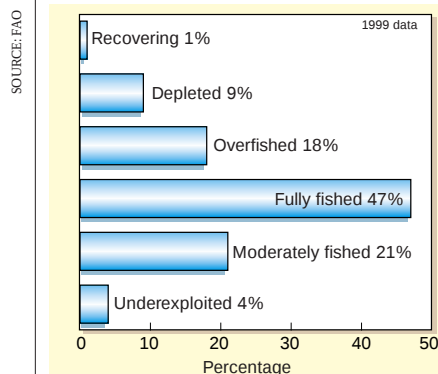
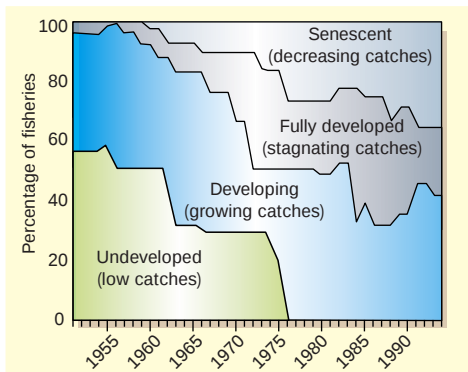
Sticking to business as usual would be a recipe for disaster, warn scientific critics of current fisheries-management practices. According to global statistics compiled by the United Nations' Food and Agriculture Organization (FAO) in Rome, the number of stocks that are being overexploited is high, and rising (see graphs, opposite). In fact, the FAO may be painting an unduly optimistic picture, having for years incorporated inflated figures that overestimate the health of China's fisheries¹. If current trends continue, claims Daniel Pauly, a conservation biologist at the University of British Columbia in Vancouver, fisheries could collapse throughout most of the world within a few decades².

Pauly is one of the foremost critics of the 'single-species' stock models that are used to calculate 'safe' allowable catches. The models can be labyrinthine in their complexity, incorporating hundreds of parameters. At their heart, however, they all rely on assessing the size and age structure of fish populations on the basis of data from experimental fishing cruises and commercial catches. The routine work of government fisheries laboratories consists largely of compiling

S. PAULY



Rocking the boat: Daniel Pauly (rear) is a vocal critic of 'single-species' modelling of fish stocks.



End of the line? Many of the world's fisheries are being plundered at or beyond sustainable limits.



Dutch trawlers blockade Rotterdam harbour in protest at restrictions on cod fishing in the North Sea. But enforcing 'no-go zones' may be the only way to preserve stocks in the most exploited fisheries.

REUTERS

these data and feeding them into the models.

But factors such as a varying climate can exert a dramatic influence on fish population dynamics, obscuring the effects of fishing pressure. And the data fed into stock-assessment models can be seriously deceiving. In the case of the Grand Banks, fisheries scientists knew that stocks were declining, but were somewhat reassured by the relatively healthy catches still being landed. They were, however, neglecting to consider the fact that fishermen were spending more time at sea, with improved equipment, fishing selectively in warmer waters where the remaining fish were congregating. Across most of the banks, there was barely an adult cod to be found.

Even scientists who regularly work with single-species stock models accept that these tools have limitations. "The links between fishing pressure, environmental changes and breeding success are not sufficiently understood," says John Shepherd, a marine ecologist at the Southampton Oceanography Centre, and formerly senior scientific fisheries adviser to the British government.

One important area of doubt is how the breeding success of different fish populations changes when their populations become diminished. Ecological theory suggests that the remaining fish, competing less intensely for food, should come to sexual maturity more quickly and boost their rate of reproduction. But a phenomenon known as depensation — a reduction in reproductive

success at low population densities, caused for example by the difficulty in finding a mate — can also come into play. "A key question in fisheries science is why, after a reduction of mortality, some species recover and some don't," says Jeff Hutchings, a marine biologist at Dalhousie University in Halifax, Nova Scotia. The presence or absence of depensation could be an important factor.

A 1995 paper³, co-authored by Hutchings, provided some grounds for optimism. The researchers examined 128 fish stocks, looking at catch data to determine numbers of spawning fish, and 'recruits' — young fish that have survived to adulthood. For only three of the stocks did these data fit with a model of population dynamics incorporating depensation. "We conclude that the effects of overfishing are, at this point, still generally reversible," Hutchings and his colleagues wrote.

Today, however, Hutchings feels rather less upbeat. If overfishing is reversible, he says, the situation can't be turned around overnight. Hutchings has analysed 90 stocks worldwide, using the largest available data set: the Stock Recruitment Database maintained by his Dalhousie colleague Ransom Myers. Many of the stocks had experienced massive declines due to overfishing. With the possible exception of fast-maturing species such as Atlantic herring (*Clupea harengus*), these stocks showed little signs of recovery as much as 15 years after their collapse⁴. "The life history of species matters," says Hutchings. "Small, early-

maturing, mid-water species like herring might recover faster than late-maturing, bottom-living species such as cod."

No way back?

Analysing the same data, Hutchings has also found that there was no association between a depleted population's recovery and the extent to which it continued to be fished after the collapse⁵. What, exactly, is holding back the recovery of overexploited stocks remains unclear — depensation might still be a factor, despite the earlier indications to the contrary. But to Hutchings, these findings demand a more precautionary approach in setting catch limits to prevent stocks from collapsing in the first place. "If there is not much we can do after the damage is done, then it is an even stronger case that we should not let fish stocks fall beyond safe levels," he says.

There is also a growing awareness that the dynamics of individual fish populations need to be considered in a wider ecological context. "Changes in predator-prey interactions and in food-web structures may interlink to cause an irreversible downward spiral," says Hutchings. Fishing for one species may also cause collateral damage elsewhere in a marine ecosystem. The barndoor skate (*Raja laevis*), for instance, has been driven to the brink of extinction largely as a result of being caught incidentally by vessels trawling for cod in the northwest Atlantic⁶. And recently, marine



Sea change: the photograph on the right shows the devastation wrought by trawlers on ancient corals (left, before fishing) in the northeast Atlantic Ocean.

scientists have begun to document the damage wrought on important habitats by trawling or dredging gear, likened by some to the clear felling of terrestrial forests⁷. Seagrass meadows off Spain, which serve as spawning grounds for many fish species, have been extensively disturbed. And in February this year, researchers led by Jason Hall-Spencer of the University of Glasgow, UK, showed that trawlers have wrecked ancient coldwater corals in the northeast Atlantic⁸.

To conservation biologists such as Pauly, these findings strengthen the case for abandoning the single-species approach to fisheries management, and turning instead to the analysis of entire marine ecosystems. Refinements to single-species stock models, and to the data that are plugged into them, offer little hope of improving the “dreadful shape” that these ecosystems are in, Pauly argues.

When fisheries are considered from an ecosystems perspective, trends emerge that otherwise aren't readily evident. In 1998, for instance, Pauly and his colleagues assigned fish to different ‘trophic levels’ in marine food webs. In their scheme, photosynthetic algae represent level 1, animals that graze on these algae are level 2, their predators form level 3, and so on. Looking at FAO catch statistics from 1950 to 1994, the researchers found that the world's fishing fleets have been steadily fishing down the food web towards lower trophic levels⁹. This is worrying, says Pauly, as the trend will reduce the complexity of marine food webs, which is likely to make ecosystems inherently more vulnerable to damage².

Pauly's Sea Around Us Project, funded by the Philadelphia-based Pew Charitable Trusts, is now using the FAO's data to investigate the impact of large-scale fisheries on

North Atlantic ecosystems. One goal is to map large marine ecosystems that share common fauna and oceanographic characteristics, and to devise policies that can mitigate and reverse stock depletion and habitat destruction.

Out of bounds

Pauly argues that sustainable fishing will only be possible if protected areas are set aside in which no fishing is allowed, where fish are able to reproduce and grow to maturity in undisturbed habitats. Historical evidence provides some support for this view: fish stocks increased substantially during the Second World War, when fisheries in the North Sea came to a standstill. And stocks around Cyprus increased in the mid-1980s after the no-fishing period enforced during the summer breeding season was extended.

Make or break for Europe's fisheries

“The desperate race for fish has to stop,” declared Franz Fischler, the European Union's commissioner for agriculture and fisheries in May, launching a far-reaching proposal

to reform the EU's Common Fisheries Policy (CFP).

Directly and indirectly, Europe's fishing industry employs more than 15 million people. But Fischler wants to reduce the EU's total fishing effort by up to 60% from January 2003. He aims to cut the current fleet of almost 100,000 vessels by 10%, while forcing the remainder to reduce their activity. Subsidies worth 460 million euros (US\$450 million) for 2003–06, currently earmarked for renewal and modernization of vessels, would be redirected to pensions and retraining for fishermen.

By mid-2004, Fischler intends to set up an inspection scheme to tackle illegal fishing and the

misreporting of catches. Sick of the “annual political horse-trading” over national catch quotas, Fischler also wants to introduce management plans lasting several years, in which advice won't be twisted by competing national agendas. And to bring policy-making closer to fishermen, he aims to create regional advisory councils, to which stakeholders can submit their own ideas about fisheries management.

It's a bold plan, and one that fisheries scientists argue is necessary if Europe's fisheries are to escape destruction. Since 1991, more than 70,000 fishermen have been driven out of a job by dwindling stocks and diminishing catches — adult cod are only half as abundant in Europe's fishing grounds as they were in the 1970s. But can the man from landlocked Austria succeed where his predecessors failed, and

reconcile competing national interests to achieve a sustainable reform of the CFP?

Time and again, efforts to reduce the EU's overexploitation of its fisheries have floundered, mired by opposition from countries with large fishing industries, such as Spain, Portugal and France. And with Fischler's plan still being discussed by the EU's member states, it could yet be blocked or watered down considerably.

If Europe can't get its fisheries in order, its problems seem certain to be exported. With European fishermen increasingly looking farther afield for their catches, the EU is already paying compensation to some West African countries for fishing rights in their coastal waters.

► http://europa.eu.int/comm/fisheries/policy_en.htm



Franz Fischler aims to stop “horse-trading” over fishing quotas in the European Union.



Shared goal: Jean Guy d'Entremont wants to foster trust between fishermen and fisheries scientists.

Most encouragingly, research published last year revealed that marine reserves established off Florida and in the Caribbean Sea have increased catches in surrounding waters¹⁰. In particular, a network of reserves off St Lucia, established in 1995 to preserve important coral reefs, has increased adjacent catches by small-scale fishermen by up to 90%.

Instituting similar policies on a larger scale will require huge changes in the way in which decisions on fisheries management are made, however. Conservation biologists want to see a reversal of the traditional 'burden of proof'¹¹. Rather than erring on the side of avoiding immediate economic pain, they would like to see precautions being taken to avoid damage to ecosystems. "The public, not industry, is the owner of the resources," argues Pauly. "But so far, all forms of fisheries management have been industry-friendly to a misplaced degree."

In the long run, the industry has everything to lose if fish stocks continue to decline. And although most fishermen still tend to regard scientists as opponents who are trying to limit their ability to earn a living, rather than partners in ensuring that their industry has a future, hints of a more productive relationship are beginning to emerge. "Fishermen have realized in the last decade that there is only a limited number of fish in the ocean," says Jean Guy d'Entremont, co-founder of an informal group of Canadian responsible fishermen, and vice-chair of the Fisheries Resource Conservation Council, which advises the Canadian government.

D'Entremont is steeped in the fishing business. He skippered an 18-metre inshore trawler for 7 years, and in 1992 took over his parents' fish-processing company in West Pubnico, Nova Scotia. In his spare time, he taught himself the basics of fisheries science. Seeing all sides of the problem, d'Entremont became convinced that fishermen must become more involved in the scientific assessment of stocks. "Fishermen are the first to touch the fish," he says. "They are the ones who can tell managers and regulators straight from the horse's mouth what's out there in terms of stocks, gear and technology."

To initiate a more productive dialogue, d'Entremont organized two North Atlantic Responsible Fishing Conferences, held in March 2000 in Fraserburgh, Scotland, and in November of the same year in St John's, Newfoundland. These meetings — a third will be held next June in Yarmouth, Nova Scotia — bring fishermen from across the North Atlantic to meet with fisheries scientists and government representatives. Fishermen can swap practical knowledge and expertise — for example, on techniques for reducing incidental catches of non-target species — while sharing their perspectives on fisheries management with policy-makers and those who advise them.

Team effort

The initiative has proved mutually beneficial, d'Entremont argues. Fishermen have brought in data from remote sea areas to assist scientists with stock assessment. And regulators have received valuable feedback on the impact of new management policies on fishing communities. Most importantly, the fishermen who have taken part say that they are now less suspicious of the scientists who advise government regulators. One Newfoundland fisherman says: "Occasionally, fisheries scientists will take the time to ask me specific questions about the fishery."

Initiatives such as d'Entremont's may be particularly relevant in Europe, where competing national agendas make the adoption of sustainable fishing policies even more problematic (see "Make or break for Europe's fisheries", opposite).

Other positive signs include the growing number of nations that are adopting aspects of the FAO's Code of Conduct for Responsible Fisheries, a 1995 document that suggests legal, technical and economic arrangements for national authorities seeking to put their fisheries on a more sustainable footing. Iceland and New Zealand, for instance, have reduced their fleet sizes and are strictly enforcing total allowable catches (TACs).

The same two countries have also led the way in dividing their TACs into individual

transferable catch quotas (ITQs) for each vessel — New Zealand introduced ITQs in 1986; Iceland, after a successful experiment with its herring fishery, applied ITQs across the board in 1990. Without ITQs, fishermen race against one another to land as many fish as possible early in the season. Total catches, as a result, frequently exceed the TAC, and illegal fishing is common. With the security of ITQs, however, individual boats no longer compete as fiercely, so fishermen can spread their harvest over the season and sell any unused portions of their ITQ. The net outcome is that total catches tend to be lower.

These policies, according to analyses by the Organisation for Economic Cooperation and Development¹², have actually increased the profitability of the two countries' fishing sectors. What's more, some stocks that are fished by their vessels are now showing signs of recovery.

But will the fishing industry ever embrace the widespread introduction of large marine reserves? Despite recent progress in building bridges between scientists and fishermen, and the evidence that reserves can boost catches, many experts still can't see it happening. "Just imagine the blockades, riots and the tonnes of fish dumped on ministers' desks if they closed down half of the North Sea," says Shepherd.

Pauly believes that involving the public in the debate will be crucial. Consumer preference for products labelled as eco-friendly, for instance, could be a powerful factor in changing attitudes within the fishing industry — particularly if the public is informed of the consequences for consumer choice if fisheries are not put on a sustainable track. If the fishing fleets refuse to withdraw from large parts of the oceans, argues Pauly, future generations won't have the option of dining on cod or other familiar favourites. Instead, they'll have to make do with jellyfish and plankton. ■

Quirin Schiermeier is Nature's German correspondent.

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FAO fisheries statistics

♦ www.fao.org/fi/statist/statist.asp

Stock Recruitment Database

♦ <http://fish.dal.ca/welcome.html>

Sea Around Us Project

♦ <http://saup.fisheries.ubc.ca>

FAO Code of Conduct for Responsible Fisheries

♦ www.fao.org/fi/agreem/codecond/codecon.asp

A million-mile service

NASA wants many of its space telescopes to orbit far from Earth. So how will they be repaired if they go wrong? Tony Reichhardt investigates.

If you keep the Sun at your back and head one-and-a-half-million kilometres away from Earth, you'll hit astronomy's most sought-after location. This unassuming spot, known as L2, is set to become home to eight new space telescopes over the next 15 years, and many more could follow.

L2 has obvious attractions. It is a lagrangian (or libration) point, which means that objects stationed there are held in a fixed position relative to Earth, as a result of the combined gravitational pull of the Sun and Earth. Ground-based antennas are always in sight, and the detectors, some of which require cooling, can more easily be protected from heat given off by Earth.

Placing telescopes at L2 could also boost NASA's plans for human space flights. Astronauts have repaired and upgraded the Hubble Space Telescope four times in the past decade. If NASA decides that astronauts should service the L2 telescopes, it will have to reinvent its human spaceflight programme, which hasn't ventured beyond Earth's orbit since the final Apollo lunar mission in 1972.

Some of the possibilities were discussed this week at the World Space Congress, held in Houston, Texas. One suggestion, developed over the past two years by a group of about 100 NASA scientists and engineers known as the NASA Exploration Team (NEXT), is to build an intermittently occupied astronaut outpost at a lagrangian point in the Earth–Moon system.

A station at this point, about three-quarters of the way to the Moon, would orbit Earth in sync with the Moon (see diagram, below). More importantly, moving the telescopes from L2 to this point and back again would require relatively little energy. Gary Martin, director of the Advanced Systems Office at NASA's Office of Space Flight in

Washington, likens it to a ridge trail in the mountains. Stay on the trail, and you'll use far less energy than if you hike up and down.

The plan has yet to be fully evaluated, but it would require expensive new technologies, including vehicles to ferry astronauts to and from the outpost, and a low-thrust propulsion system to move the telescopes. The outpost would, however, have other uses. Astronauts could be dispatched to the Moon from it, and it could also be a base from which to assemble and launch an expedition to Mars.

But if telescope servicing is the main aim, NASA could avoid building an outpost, says Robert Farquhar, a veteran space-mission designer at the Applied Physics Laboratory in Laurel, Maryland. Farquhar, who also presented his ideas in Houston, believes that the telescopes could be moved from L2 into a very high Earth orbit and serviced there.

Safety catch

He and others say that direct missions to the L2 telescopes may also be possible. At distances greater than about 40,000 kilometres from Earth, the planet's magnetic field no longer protects astronauts from the dangerous charged particles emitted by the Sun and other sources. To play it safe, NASA would keep long-distance human missions as short as possible, although it has made radiation-protection research a priority on the International Space Station. Round trips to L2, which would take at least a month, could be possible if the agency solves this problem.

So will any of the first generation of L2 telescopes require human involvement? One, the Microwave Anisotropy Probe, designed to measure background levels of microwave radiation, is already in place. But like the others now on the drawing board, such as NASA's James Webb Space Telescope, the successor to



Will astronauts be able to reach and service equipment farther away than the Hubble telescope?

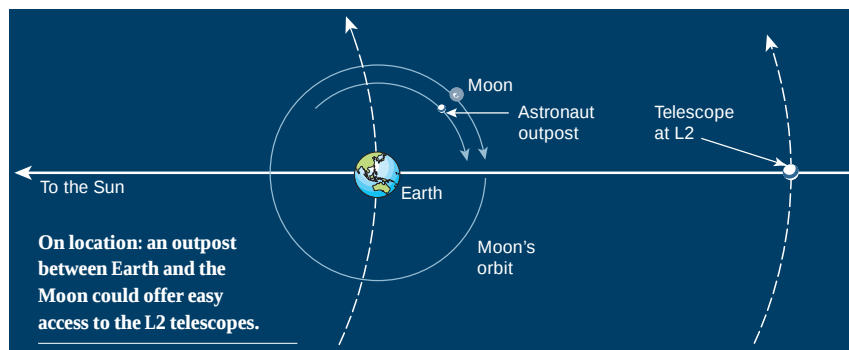
Hubble (see *Nature* **419**, 235–236; 2002), it was not built to be serviced by astronauts.

"There's nothing better than an astronaut in the loop," says Charles Beichman of NASA's Jet Propulsion Laboratory (JPL) in Pasadena, California, chief scientist for the Terrestrial Planet Finder (TPF), an L2 telescope that should start hunting for Earth-size planets in the middle of the next decade. "But it will be a significant investment. We don't want to stop what we're doing while waiting for them."

The situation may have changed, however, by the time larger, more complex telescopes are ready to follow the TPF. The NEXT team, for example, is honing its plans by assessing the needs of the Dual Anamorphic Reflector Telescope, currently under study at JPL.

This large but lightweight infrared telescope is unlikely to be launched within the next 15 years. But if NASA does decide to use astronauts to service such telescopes, it would present a historic opportunity to align the agency's science and human spaceflight programmes, says Wesley Huntress, NASA's former associate administrator for space science who now directs the Carnegie Institution of Washington's Geophysical Laboratory. From Apollo onwards, astronaut missions have been dictated by space engineering capabilities and politics. If human telescope servicing is needed, says Huntress, scientists could find themselves setting the agenda.

Tony Reichhardt writes for *Nature* from Washington.



Corporate ethic is now undermining universities too

Adoption of money-centred business practices leaves academia open to same abuses.

Sir — Although I applaud the overall message of your Opinion article “After the gold-rush” (*Nature* **418**, 111; 2002), I do not share your optimism that the scandals emerging in US businesses and the resultant distortion of scientific priorities have “not done much damage to the integrity of universities or scientific institutions”.

Many US universities have begun switching their administrative processes to follow a more corporate model, run on a ‘star’ system similar to that of companies such as Enron. In this system individuals are promoted, not because of experience or expertise, but on perception of their so-called intelligence and charisma. Large salaries can thus be given to mid-level administrators and a few faculty members sympathetic to ‘reforms’ and ‘streamlining’ (see www.workplace-gsc.com for a discussion of this issue).

One example of this practice is the use

of new personnel policies based on business principles and maximizing revenue. Such policies (including attempts to end tenure) can effectively create deregulation within an institution. The resultant breakdown of due process leaves only the courts to resolve internal matters, a phenomenon which is also being seen in corporate scandals.

Another issue is that of shaky accounting, in which department chairs inflate the amount of grant money brought in by their faculty. They may list grants received by a group of faculty as if each member had received that amount, for example, or bring in badly paid temporary lecturers to replace tenured faculty members who have left.

Attempts to raise revenue have caused some university administrators to appoint to senior positions former executives from corporations embroiled in accusations and

scandals. Some university presidents serve on the boards of directors of companies that may have competing financial interests or may be subject to investigation of their business practices. Under circumstances such as these, the association between university administrators and businesses is too close for comfort.

Practices and philosophies that have emerged from the economic boom and subsequent bust are only now beginning to harm the integrity of scientific institutions. Academic scandals are less well known because they do not (yet) involve the large amounts of money reported in corporate scandals. But the basic elements of serious scandals are likely to be as common in US universities as they are in US business.

Raymond Pierotti

President, KU American Association of University Professors, Division of Biological Sciences, University of Kansas, Lawrence, Kansas 66045, USA

Indian biotech sets a constitutional challenge

Sir — Immediately after publication of your News story “Transgenic cotton gets mothballed after protests” (*Nature* **418**, 716; 2002), the agriculture minister of Karnataka retracted the state’s ban on Bt cotton seeds on the grounds that only the central government can make such a decision (*The Hindu*, 16 August 2002). The state can advise central government on whether to impose a ban, if evidence can be shown of adverse impact on animal (including human) and plant health.

In this case, the state minister banned Bt seeds in the light of concerns about their environmental impact after he had been briefed by protesters citing a Greenpeace report about Bt cotton in China. However, the minister had not at that time been informed about criticisms of this report by two Chinese scientists, who called it “garbled and biased” (see the Biosafety Information Network and Advisory Service, <http://binas.unido.org/binas>), hence his swift retraction of the ban.

This controversy represents a constitutional challenge, as the Indian constitution makes the environment the responsibility of central government and agriculture that of individual states. The state of Karnataka can regulate and restrict any executive and legislative process initiated by central government in the domain of agriculture. However, the introduction, development and commercialization of genetically

modified plants are governed by centrally enacted environment laws in India.

This type of confusion can only hamper the advance of biotechnology and the development of agriculture, and needs to be resolved by bringing agriculture under the constitution’s Concurrent list. This would eliminate the possibility of disputes because, in cases of disagreement about items on that list, central government legislation prevails over that of the states.

Bhagirath Choudhary

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All shipshape at navy lab

Sir — As a 37-year employee of the US Department of the Navy, I have had a 360° view of the Naval Research Laboratory (NRL) mentioned in your News story “Senators attack Pentagon over weak state of defence research” (*Nature* **418**, 711; 2002). It is my personal view that she is still a vigorous centre of excellence in science and technology related to US naval services. The shrinking of the workforce noted by Vice Admiral Paul Gaffney results from declining funds allocated for in-house research, and the expectation that the commercial products of the ‘new economy’ will replace the specialized basic knowledge and engineering skill necessary to buttress US naval superiority.

The perception that the private sector

can provide superior innovation and be a more generous source of compensation for the stellar contributor or daring innovator rose with the US stock market and may be collapsing along with the Internet bubble. Revelations of accounting that is more creative than accurate, the ‘Dilbertization’ of engineering and programming with meaningless and unrewarding jobs, recent terrorist attacks on the United States and economic uncertainty are all making public service seem attractive again.

NRL’s ‘demonstration programme’ for personnel already allows competitive offers to be made to all but the top few per cent of researchers. The lab’s excellent facilities, access to actual data, continuing education opportunities, creative environment and sense of mission all help maintain the continuity of the world-class research programmes over the decades that it sometimes takes to move first-principles research to deployed capability. Restoring NRL’s workforce to previous levels will be necessary to meet the needs of knowledge-centred warfare. This will require greater support and wider appreciation of the distinction between useful research and commercial product development.

NRL is not “all at sea” and is neither dead nor in need of revitalization, but is firmly perched on the left bank of the Potomac, flush with qualified applicants, ready to serve the national defence of the United States.

R. A. LeFande

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The birth of molecular biology

How biophysicists and biochemists in the 1950s shaped a new science.

Designs for Life: Molecular Biology After World War II

by Soraya de Chadarevian

Cambridge University Press: 2002. 444 pp.

£35, \$55

Vernon M. Ingram

The history of the genesis and development of the molecular-biology group at Cambridge University, UK, under Max Perutz is endlessly fascinating. Why did it begin? Why in Cambridge? Why at that time? And why these particular scientists?

Soraya de Chadarevian presents a historian's account of the conception and birth of the group that founded what is arguably the reigning movement in modern biology. She describes the circumstances and tactics needed to enable the field of molecular biology to mature from a child to become an adolescent, with predictable awkwardness, and then to achieve adulthood. Throughout gestation and childhood it was nurtured by Perutz, who had a clear vision of the next essential step in the development of biological science.

Perutz, who died earlier this year, set out to solve the chemical structure of haemoglobin, the protein molecule that carries oxygen in vertebrates. He took from John Desmond Bernal and Lawrence Bragg the notion that the young science of X-ray crystallography could be used not only to find the chemical structures of small molecules such as salts and sugars, but also the structure of enormously complex molecules such as haemoglobin. Many people told him he was crazy, that the task was impossible; however, he succeeded after some 25 years. De Chadarevian gives a clear account of this story.

In the late 1940s and early 1950s, Perutz attracted a nucleus of remarkably able young collaborators. His single-minded devotion to the task and his personality were key to this collaboration coming together. There was John Kendrew, who solved the structure of the muscle protein myoglobin; Francis Crick and James Watson, who solved the structure of DNA; Tony Broad, the engineer who made the most powerful X-ray machine in the world; Hugh Huxley, who (with Jean Hanson) solved the molecular mechanism of muscle contraction; and, a little later, Sydney Brenner, who with Crick founded much of modern molecular genetics. I was fortunate to be an early member of the group (1952–58), working as a protein chemist, helping the X-ray crystallographers, and studying the defect caused by the sickle-cell anaemia mutation. Perutz was mentor to the whole group.



Protein pioneers: (from left) Hugh Huxley, John Kendrew, Max Perutz, Francis Crick, Fred Sanger and Sydney Brenner were the driving force behind the Laboratory of Molecular Biology in Cambridge.

The excitement about our work was palpable; it permeated every conversation and dominated our leisure time. However, the book fails to capture this excitement. This is unfortunate, because we were spurred on and held together by an obsessive desire to understand the molecules of life — proteins and nucleic acids. In *Designs for Life*, the historian eclipses the storyteller.

Also lost is the spirit of intense competitiveness that we felt towards Linus Pauling and his group at the California Institute of Technology, and the X-ray crystallographers at King's College London. Although de Chadarevian gives historical credit to other groups who worked on X-ray crystallography, and protein chemistry in particular, she only touches on the crucial importance of knowing the amino-acid sequences of myoglobin and haemoglobin when progressing from a crude to a detailed structure of these proteins. This information was developed in the United States, and was not available earlier to Perutz and Kendrew.

The early X-ray crystallographers' use of existing and new technologies to solve major biological problems is well described in this book — they were “the right men at the right time”. Also fascinating is the crucially important and parallel development of digital computing in the Cambridge University Mathematics Department next door. There was a difference in personality between Perutz and his pupil Kendrew. The latter

embraced and spearheaded the development of computers, but he had to be defensively careful about their use because of Perutz's early scepticism about the accuracy of the new method.

Designs for Life deals well with certain important areas of history. The author links the timing of the appearance of the early Medical Research Council (MRC) unit in Cambridge to the availability of new technologies developed during the war in Britain and the United States, and to the availability of young and eager scientists who had had maturing experiences during the Second World War. The early group, led by Perutz, used their success in solving these incredibly difficult structures to publicize the new science of molecular biology by television, radio and newsprint. They encouraged the development of similar research groups elsewhere, and taught molecular biology to a new generation of students from many countries.

Especially detailed is de Chadarevian's account of the politics involved in expanding the group to the size of an institute, large enough to be varied and self-sustaining. The difficulties encountered were both academic and governmental. Although there is much repetition of this theme, there is also a great deal that is interesting.

The group's expansion was helped enormously by the arrival of Fred Sanger, a protein biochemist who went on to win two Nobel prizes — he was the first to establish

the complete chemical structure of a protein, insulin, and he later invented the current method for sequencing the genome.

In the late 1950s and early 1960s, the vigorous drive to establish new molecular-biology departments was in full swing in the United States but had barely begun in Britain. As a result of the reluctant atmosphere at Cambridge University, the hoped-for expansion did not occur within a university department. There were delays, prevarication and disappointments before the group arrived at their large new MRC site on the outskirts of Cambridge. Unfortunately, this was far away from the university, so the researchers were not integrated into its teaching and collegiality. This enforced separation was in part responsible for the desire of so many of the group to leave the MRC laboratory for teaching positions elsewhere. It is interesting to speculate whether a true integration into Cambridge University would have prevented the exodus.

Much space in this book is devoted to the discovery and worldwide expansion of structural protein biochemistry, and rightly so. It is therefore surprising that less attention is paid to the discovery of the Watson–Crick model of DNA. The impact of their double-helix structure was tremendous and lasted for years. Even more than the great influence of protein-structure determination, the DNA model and its consequences were crucial to fashioning modern molecular biology.

In describing the development of molecular biology, de Chadarevian pays some, but not enough, attention to the vital role of scientists such as Erwin Chargaff, William Cochran, Rosalind Franklin and Linus Pauling, not to mention the other American and French groups. After all, truly great advances in biology are built on the work of others — to give them credit would not diminish the achievements of Watson, Crick and Brenner.

Perhaps de Chadarevian should have paid more attention to the influence of Crick and Brenner on the development of the new and exciting fields of molecular genetics and protein synthesis. Their work and the publicizing of their ideas made molecular biology the lingua franca of modern biology. Even the new generation of engineers feel the urgent need to learn this new treatment of biology.

The author deliberately focuses on the (unique) instance of the MRC Unit for the Molecular Structure of Biological Systems, housed in the Cavendish Laboratory, Cambridge, and its direct descendant, the MRC Laboratory of Molecular Biology. The account of these units' relationship to government, to private funding agencies and to the university makes an interesting and valuable book. One must realize, however, that the narrow focus does not imply that the group was entirely self-contained — we

depended, at the time, on the outside world for scientific nourishment and for funding. In short, de Chadarevian's historical account is recommended to all who are interested in the development of molecular biology. ■

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Peering through the smoke

Understanding Marijuana: A New Look at Scientific Evidence

by Mitch Earleywine

Oxford University Press: 2002. 344 pp. \$29.95

Raphael Mechoulam

There seems to be an insatiable appetite for information on marijuana. The 'books in print' file on my computer lists 416 books under 'marijuana', for example. Is such a deluge justified? Probably not. Does this book by Mitch Earleywine merely add to it, or is it really, as the subtitle claims, a "new look" at the scientific evidence? It certainly brings together a considerable amount of information on cannabis in many fields, and does so very well, but a new look it is not.

In the past two decades there has been a renaissance in cannabis research, mostly in chemistry and biology. Cannabinoid receptors have been identified and endogenous cannabinoids have been isolated from the brain and the periphery. The endocannabinoids have been found to take part in processes in almost all of the major physiological systems. Most of the work has focused on the nervous system, but there have been advances in our understanding of their role in the immune, reproductive, cardiovascular and digestive systems. Memory, pain, inflammation, appetite and suckling are some of

the physiological processes in which cannabinoids are involved. By contrast, most other aspects have been sadly neglected.

The endogenous cannabinoids, although now known about for about a decade, have never been administered to healthy human beings, let alone patients. Can we compare this snail's pace with that witnessed with the hormones insulin and cortisone, for example, when, decades ago, clinical research led to major advances in therapeutics within months of their discovery? The only cannabinoid drugs on the market are delta-9-tetrahydrocannabinol (THC), the psychoactive constituent of cannabis, and nabilone, a more active synthetic drug with a similar profile. Both were introduced about 15 years ago. Even cannabidiol, a non-psychoactive, non-toxic cannabis constituent with known anti-oxidative, anti-inflammatory, anti-nausea and anti-epileptic properties, has yet to be adequately evaluated in patients.

This deplorable situation has hampered advances in many fields, particularly in psychology. There is a limit to what animal work can teach us about human emotions, cognition and behaviour. Until we learn more about the endocannabinoid system in humans, a "new look" at the field is bound to be premature.

Two years ago, Oxford University Press published *The Science of Marijuana* by Leslie Iversen (for a review, see *Nature* **407**, 18–19; 2000), an excellent book in which the emphasis is on the biological aspects and medical uses of marijuana. Earleywine, on the other hand, while also addressing these aspects, devotes most of his book to the psychological and social aspects of marijuana use. Unfortunately, these aspects have not been part of the scientific renaissance of recent years. Although the available material is thoughtfully and critically discussed, I was left with a feeling of *déjà vu*.

Earleywine, as befits a clinical psychologist, believes in both statistics and common



High tea? Cannabis cafés have focused attention on the controversy surrounding the use of marijuana.

B. MARGOT/AP

sense. On the basis of a detailed analysis of the literature, he draws several conclusions. The first is that the assumption that marijuana serves as a 'stepping stone' to the use of other drugs is not valid: "Children may benefit more from spending their time in pursuing a classical education than listening to a police officer tell them to abstain," he declares. Second, he argues that although acute marijuana intoxication impairs the ability to perform some cognitive tasks, chronic heavy use probably does not affect gross intellectual functioning, although it may affect the performance of complicated tasks. Third, he reaches the conclusion that marijuana alters perception and 'slows' time: "The senses generally seem more appealing and interesting, despite laboratory evidence that they be actually impaired." Finally, he finds no evidence that marijuana causes a lack of motivation, reckless driving or aggression. These conclusions mirror the attitudes of most researchers in this field.

The chapter on law and policy is well balanced, and Earleywine considers the moral and pragmatic arguments advanced by both the prohibitionists and the proponents of legalization or decriminalization before concluding that "this is one area where more research will not have as much impact as changes in political climate".

Understanding Marijuana was published before the recent report by the Canadian Senate committee on cannabis, whose superb analysis of the problem and common-sense proposals fit with the overall view of Earleywine. The Canadian committee recommends an approach that "is neither one of total abstinence nor an indication of abandonment but rather a vision of the role of the State and criminal law as developing and promoting but not controlling human action". It is based on their conclusion that "used in moderation, cannabis in itself poses very little danger to users and to society as a whole, but specific types of use represent risks for users... even if cannabis were to have serious harmful effects, one would have to question the relevance of using the criminal law to limit these effects". I believe this is a key point that Earleywine tries to stress with regard to law and policy.

The chapters on pharmacology, health effects and medical marijuana are also a good summary of these fields, although there are mistakes and omissions. Cannabidiol is not psychoactive, as stated, and does not become delta-9-THC as the marijuana plant matures; cannabinoids probably do not suppress vomiting via cannabinoid receptors, as cannabidiol, which does not bind to these receptors, is also an anti-nausea agent; and Earleywine does not mention the potent effects of cannabidiol in models of rheumatoid arthritis and anxiety. Surprisingly, neither the identification of THC as the psychoactive cannabis constituent nor the

cloning of the CB1 cannabinoid receptor is referred to in the otherwise very good and extensive reference list.

Despite minor mistakes and omissions, though, Earleywine has written a well balanced, up-to-date, non-specialist book that should appeal to a wide audience. ■

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More on marijuana

Biology of Marijuana: From Gene to Behavior

edited by Emmanuel S. Onaivi
Taylor & Francis, £95, \$150

A protean overview

The Physics of Phase Transitions: Concepts and Applications

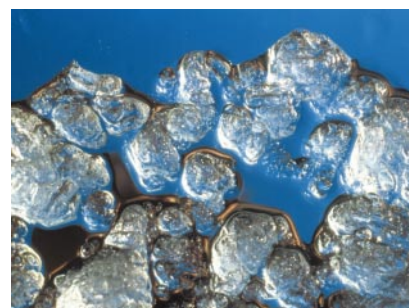
by P. Papon, J. Leblond & P. H. E. Meijer
Translated from the French by S. L. Schnur
Springer: 2002. 397 pp. £49, \$74.95, 69.95 euros

Robert W. Cahn

Phase transformations, flexibly regarded, are central not only to materials science but also to various subfields of physics, and spill over into physical chemistry. Two of the authors of this survey (the third is at a US university) teach at the Ecole Supérieure de Physique et Chimie Industrielles de la Ville de Paris, France, and the twin pulls of its constituent disciplines are clear to see — as is the pervasive influence of Pierre-Gilles de Gennes, the Nobel laureate who headed this *grande école* for many years.

Pierre Papon and his co-authors succeed in covering a much wider range of transitions than I have ever seen in one book before: solidification and melting; critical gas-liquid behaviour; the glass transition; gelation in 'soft matter' and biopolymers; collective (critical) phenomena in solids and liquids — this last incorporating magnetism, ferro-electrics, liquid crystals in much detail, superconductivity and superfluidity; a ragbag of 'nanostructures' (quasicrystals, colloids, emulsions and martensite); monolayers and Langmuir-Blodgett films; with a grand finale focused on geomaterials, plasmas and the ocean-atmosphere system.

Each theme begins with a careful theoretical account, firmly centred on classical thermodynamics and statistical mechanics; readers are expected to be well versed in both of these fields. Broad topics such as fractals and percolation theory are helpfully summarized; others, such as the theory of phonons, are not. Theory is bolstered in each chapter with an array of theoretical problems (answers provided), and appendices cover



Losing its cool: ice undergoing a phase transition.

particularly difficult topics such as renormalization group theory. With the theory out of the way, a range of applications of each theme is summarized in each chapter.

Experimental approaches to the many forms of transformation receive rather less attention, but enough is said (about David Turnbull's droplet method of studying homogeneous nucleation, for instance) to give an impression of how numbers can be put into the theory. Applications of quantum theory are notable for their paucity here; such matters as the use of electron theory to estimate the free energies of rival metallic phases are not treated. There are no literature references for individual pieces of theory or experimental research, but each chapter is provided with a useful select bibliography.

Many links between apparently distinct phenomena are made clear. The remarkable breadth of this book is its principal claim to distinctiveness but it also, inevitably, results in superficial accounts of some of the themes. Transformations in metals and alloys, and in ceramics, receive limited attention. Because of this, but particularly because of the high level of expertise in statistical mechanics expected, the book will find only limited use in the education of materials scientists. Yet physics students and those who labour at the ill-defined interface of physical chemistry and chemical physics will find much of educational benefit here.

The translation from the 1999 French edition is very competently done. There is just one repeated linguistic flaw, which I frequently find in anglicized versions of French prose: 'important' in the sense of 'significant' and in the other French sense of 'large' are confused; and many large things are claimed to be important. Maybe Frenchmen and Texans have more in common than either might like to believe.

Overall, we have here a treatment of strikingly wide perspective, and many readers who may not be motivated to work right through the book will find individual chapters interesting and instructive. I defy anyone who is interested in phase transformations not to learn something from this book. ■

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An eye on the future

Lawrence H. Goulder and
Robert N. Stavins

Decisions made today usually have impacts both now and in the future. But in carrying out policy evaluations to help decision-makers, economic analysts typically discount future impacts. In the environmental realm, many of the future impacts are benefits from policy-induced improvements. Thus, in the environmental-policy context, future benefits (as well as costs) are often discounted.

This is controversial, partly because discounting can seem to give insufficient weight to future benefits and thus to the well-being of future generations. But does it actually shortchange the future? As economists, we have often encountered scepticism about discounting, particularly from non-economists. Some of this scepticism seems valid, yet some reflects misconceptions about the nature and purpose of discounting. By examining

here how discounting affects the evaluation of environmental policies, we hope to clarify this concept.

It helps to begin by considering the use of discounting in private investments. Here, the rationale stems from the fact that capital is productive — money earns interest. Consider a company deciding whether to invest \$1 million in the purchase of a copper mine, and suppose that the most profitable strategy involves extracting the available copper three years from now, yielding revenues (net of extraction costs) of \$1,150,000. Would investing in this mine make sense? Assume that the company has the alternative of putting the \$1 million in the bank at 5% annual interest. Then, on a purely financial basis, the company would do better with the bank, as after three years it will have \$1,157,625 ($\$1,000,000 \times (1.05)^3$), compared with only \$1,150,000 if it invests in the mine.

Future returns

We compared these alternatives by compounding to the future the up-front cost of the project. It is mathematically equivalent to compare the options by discounting to the present the future revenues or benefits from the mine. Discounting offers a quick way to check whether the return on a project is greater or less than the interest rate by taking future revenues and translating them into present units, using the 'alternative rate of return' (the bank's rate of interest in our example) as the discount rate. So the discounted revenue in this case is \$1,150,000 divided by $(1.05)^3$, or \$993,413 — less than the cost of the investment. Thus, the project would not earn as much as the alternative of putting money in the bank. If the discounted revenue exceeded the cost of the project, then the project would yield a higher return than the bank, and the company would be better off investing in the mine.

This simple example suggests a general formula to determine whether an investment offers a return that is greater or less than the alternative of putting money in the bank. Suppose a project involves benefits (revenues) and costs over a time span from the present (time 0) to T years from now. Let B_t and C_t refer, respectively, to the benefit and cost t years from now, and let r represent the annual rate of return on a standard investment. The present value of the net benefit (PVNB) is given by

$$\text{PVNB} = \sum_{t=0}^T (B_t - C_t) / (1 + r)^t$$

If this value is positive, the project will yield

Discounting

How economists' controversial practice of discounting really affects the evaluation of environmental policies.

a return that is higher than the market interest rate.

Discounting translates future sums of money into equivalent current sums; it undoes the effects of compound interest. It is not aimed at accounting for inflation, as even if there were no inflation it would still be necessary to discount future revenues to account for the fact that a dollar today translates (through interest) into more dollars in the future.

Sums for society

Can the same kind of thinking be applied to investments made by the public sector for the benefit of society? Consider the following hypothetical public-sector investment: a potential climate policy. Our purpose is to convey key issues in the starkest terms, so we will intentionally oversimplify some aspects of what follows. Suppose that a policy, if introduced today and maintained, would avoid significant damage to the environment and human welfare 100 years from now. The 'return on investment' is the avoidance of future damage to the environment and to people's well-being. Suppose that this policy costs \$4 billion to implement, and that this cost is borne in its entirety today. Suppose also that the beneficial impacts — avoided damages to the environment — will be worth \$800 billion to people alive 100 years from now. Should the policy be implemented?

The answer will depend, of course, on the evaluation criteria used. Consider first the criterion of whether the winners have the potential to compensate the losers and still be no worse off. For this condition to be met, the benefit to the winners, after being translated to equivalent dollars, needs to be larger than the losses of the losers. After compensation from winners to losers, the policy would yield what economists call a 'Pareto improvement': some individuals would be better off, and no individual would be worse off.

Are the benefits great enough that the winners could potentially compensate the losers and still be no worse off? Here, discounting is helpful. If, over the next 100 years, the average rate of interest on ordinary investments is 5%, a gain of \$800 billion to people 100 years from now is equivalent to \$6.08 billion today. (Equivalently, \$6.08 billion today, compounded at an annual interest rate of 5%, will become \$800 billion in

H. ARMSTRONG ROBERTS/CORBIS



Laying a golden egg? Discounting allows potential investors to weigh up their potential returns.

100 years.) The project satisfies the principle of potential compensation if it costs the current generation less than \$6.08 billion.

Because the up-front cost of \$4 billion is indeed less than this figure, the benefit to future generations is more than enough to offset the cost to the current one. More generally, a positive PVNB means that the policy has the potential to yield a Pareto improvement. More realistic policies involve costs and benefits that occur at all points in time. For these policies, discounting serves the same purpose, as we convert costs and benefits from various periods into their equivalents at a given time (such as the present, for example).

Applying a discount rate does not mean giving less weight to the welfare of future generations. Rather, the process simply converts the (full) values of the impacts that occur at different points of time into common units. In our example, the full benefit to future generations is translated into a current monetary sum, which then allows us to compare this benefit with the full cost to the present generation.

Winners and losers

Even if one accepts the idea of discounting as a mechanism to translate impacts into equivalent monetary units, one might be uneasy about PVNB analysis, which is based on the potential Pareto improvement (PPI) criterion — whether the winners from a given policy could compensate the losers and still be better off. If a policy's benefits exceed its costs, and compensation is introduced so that no one is worse off, then the attractiveness of the policy seems clear. But if compensation is not actually made, the appeal seems considerably weaker.

In our climate-policy example, discounted benefits to future generations will exceed the loss to the current generation, so the potential exists for a Pareto improvement — the PPI criterion is met. But if future generations do not actually compensate the present one, is it still appropriate to enact the policy? Perhaps so, but many would argue that if actual compensation cannot be made, a positive PVNB has less merit as an evaluation criterion.

Suppose a proposed climate policy fails the PPI test — the future benefits are not large enough to offset current costs. Do current generations nevertheless have an obligation to undertake the policy? They might. The PPI criterion deserves to be given

weight, but in almost all policy evaluations — especially when compensation is not actually carried out — it is important to consider other evaluation criteria, as there are bound to be some cases in which there are compelling reasons for adopting a policy even when the PPI criterion is not satisfied, or for rejecting a policy even when it is.

Should a lower discount rate be used to incorporate considerations of intergenerational equity more fully in the PVNB calculation? Suppose that, when the market interest rate is used for discounting, a policy that would benefit future generations fails to generate a positive PVNB. Using a lower discount rate would give greater weight to future benefits (and costs), possibly making the PVNB positive. Such adjustments are problematic, however: they blur the distinction between the PPI (efficiency) criterion and other legitimate policy-evaluation criteria, such as distributional (in this case, intergenerational) equity. In evaluating policies, it seems better to use the market interest rate so that the PVNB calculation provides a meaningful indication of whether the PPI criterion is satisfied, while at the same time judging intergenerational fairness by direct examination.

Even if one accepts the use of the PPI criterion and discounting in principle, estimates of PVNB are necessarily imprecise. There is uncertainty about the denominator — the discount rate. Theoretically, this should reflect the market interest rate but, of course, future market rates are impossible to predict. There is also considerable uncertainty about the elements in the numerator — the benefits and costs that current and future generations will experience from a policy that is introduced today. This uncertainty is derived both from scientific uncertainty about the biophysical impacts of policies and from uncertainty about future generations' tastes and preferences — how much they will value the biophysical impacts.

Much scepticism about discounting and, more broadly, the use of benefit–cost analysis, is connected to these uncertainties. Consider the difficulties of ascertaining, for example, the benefits that future generations would enjoy from a regulation that protects certain endangered species. Some of the gain to future generations might come in the form of medical products (such as serums or vaccines) derived from the protected species, but such future impacts are impossible to predict. Moreover, benefits reflect the value

that future generations will attach to the protected species — the enjoyment of observing them in the wild or just knowing of their existence. But how can we predict future generations' values? Economists and other social scientists try to infer them through surveys (such as the contingent valuation method) and by inferring preferences from individuals' behaviour. But these approaches are far from perfect, and at best they indicate only the values or tastes of people alive today.

The uncertainties are substantial and unavoidable. They do not invalidate the use of discounting or benefit–cost analysis, but they do oblige analysts to acknowledge them in their policy evaluations. It is crucial to evaluate policies using a range of values for discount rates and for future benefits and costs. We should have less confidence in a project for which the sign of the PVNB is highly sensitive to the discount rate or to small changes in projected future benefits and costs, compared with a project with a PVNB that is not very sensitive to these elements.

The discounting debate

The application of discounting to environmental-policy evaluation is controversial, partly because of misunderstanding outside the economics community of what discounting actually does, which is to translate the values of future impacts into equivalent values in today's monetary units. The PPI criterion, which provides the rationale for discounting and calculation of the PVNB, deserves weight in evaluating environmental policies, although it is also important to consider other criteria (such as distributional equity), especially when the potential harm to 'losers' is substantial. Moreover, it is crucial to acknowledge any uncertainties about benefits, costs and interest rates. Some may argue that these complications invalidate PVNB calculations, but in our view such calculations — when carefully executed and thoughtfully interpreted — can provide useful information for making environmental-policy decisions. ■

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Uncertainty is unavoidable. This does not invalidate the use of discounting or benefit–cost analysis, but it does oblige analysts to acknowledge it in their policy evaluations.

Into the heart of darkness

Karl Gebhardt

The Milky Way, like other galaxies, is thought to harbour a black hole at its centre. The remarkable observation of a star in close orbit around the Galactic Centre is the first firm evidence that this is so.

Reporting on page 694 of this issue, Schödel *et al.*¹ describe the first observation of a nearly complete orbit of a star around the black hole at the centre of our Galaxy, the Milky Way. We have long been able to trace the movement of planets in our own Solar System, and astronomers are now beginning to observe planetary orbits in other stellar systems², but this is the first time that an orbit has been detected on the galactic scale. The sheer size of galaxies normally makes the detection of such movement impossible within a human lifetime. For example, our own Sun takes 230 million years to circle the Milky Way. The star that Schödel *et al.* report on will complete its orbit around the central black hole of our Galaxy in a mere 15 years — lightning speed on the grand, slow scale of the Universe.

That we are now able to watch the orbit of a star across our Galaxy is only one of several important implications of Schödel and colleagues' findings. For many years now, astronomers have been reporting that supermassive black holes — more than a million times the mass of the Sun — exist in nearly every galaxy³; scientists now even have data that suggest that black holes also occupy the centres of smaller stellar systems called globular clusters^{4,5}. But despite decades of research and discovery, no one had found

Figure 1 Journey to the centre of the Galaxy. This image, taken by the Very Large Array of ground-based telescopes at radio wavelengths, shows a bright source at the centre of the Milky Way that was thought to surround a black hole. From their observations of a star in orbit around the Galactic Centre, Schödel *et al.*¹ conclude that there is indeed a supermassive black hole in this region. The structure known as the Galactic Centre Radio Arc (upper left) is believed to be generated by hot plasma flowing along lines of magnetic field.

conclusive evidence that supermassive black holes exist.

The problem was that astronomers had never been able to observe the centres of galaxies closely enough to rule out other possibilities, such as a collection of neutron stars masquerading as a central black hole⁶. But these new data probe the Galactic Centre more closely than ever before. The matter density that Schödel *et al.*¹ infer from the details of the star's orbit is inconsistent with the presence of neutron stars, or other more exotic objects. The only compelling explanation is that there is a supermassive black hole lurking there. These results are the best evidence yet that supermassive black holes are not just theory, but fact.

The technique used by Schödel *et al.* to measure the stellar orbit is also impressive. Their success shows the true power of a relatively new observing tool, adaptive optics imaging⁷. Starlight reaching ground-based telescopes becomes blurred as it travels through the Earth's atmosphere. In

an adaptive optics system, the distortions in the incoming beam are measured, and electronic signals sent to a deformable mirror. In response, the mirror can rapidly change its shape to correct for those distortions as it reflects the starlight. The blurring effects of the atmosphere are removed almost completely from the data and thus ground-based observations can be as sharp and informative as those from space-based telescopes.

Using this new technology, we are now able to see images that are up to 20 times sharper than they once appeared, making it possible to differentiate individual stars in the crowded stellar regions at the centre of the Milky Way (Fig. 1). Faint stars that were practically invisible can now be isolated, and more stars in orbit at the very centre of our Galaxy could be found. Measuring the orbits of these stars should provide even stronger evidence in favour of a black hole, and it should eventually be possible to test the predictions⁸ of general relativity using stars that

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pass even closer to the black hole. The power of adaptive optics may also enable us to determine how material is funnelled into a supermassive black hole or, in the case of the black hole in the Milky Way, why so little matter is actually consumed by it⁹.

Although the main point of interest for the layperson may be the proof that black holes are real, scientific research is more concerned with gathering the best possible data and determining the most accurate results. The black hole in our Galaxy does not have the best-determined mass, but with continued observations, following those of Schödel *et al.* and others¹⁰, it will soon be the best constrained. The exact value of its mass has important implications for understanding how our black hole compares with those at

the centres of other galaxies¹¹. We still have a way to go, but for this black hole the future is bright. ■

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Microbiology

All in the packaging

Edward F. DeLong

Certain bacteria generate highly toxic intermediates as part of their metabolism. Membranes with an unprecedented lipid composition and structure apparently meet the need for containment.

Lipid membranes form the major barrier that distinguishes ‘self’ from ‘non-self’, and they are essential for keeping cellular components inside a cell and toxins outside it. They are also active participants in the energy and transport processes necessary for cell growth and survival. The cells of eukaryotes (all animals, plants, protists and fungi) also house membrane-bound structures called organelles, which have specialized functions — for example, energy production in mitochondria and photosynthesis in chloroplasts.

At first glance, the fundamental composition of membrane lipids among life’s three domains (eukaryotes, bacteria and archaea) seems much the same and rather simple. In eukaryotes and bacteria, the chemical make-up of the predominant membrane lipids follows a common theme. Generally, they consist of two fatty acids, each joined by an ester group to a phosphorylated glycerol backbone. In the archaea, the arrangement is somewhat different, consisting of two isoprenoid units bound to a glycerol backbone by a very stable ether linkage.

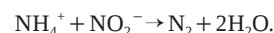
Now, on page 708 of this issue, Damsté and collaborators¹ report on the presence and structure of bizarre bacterial lipids never before seen in nature. These lipids, called ladderanes, occur in organelle-like structures in an unusual bacterium that makes its living by consuming ammonia in the absence of oxygen.

The bacterial lipids discovered by Damsté *et al.*¹ deviate considerably from those normally found in bacteria and archaea.

Some of the lipids are bound to a glycerol backbone by an ether linkage, which is unusual but not unprecedented in bacteria. More striking are the core lipid structures that have never been seen before in any organism. One contains three consecutive four-carbon (cyclobutane) rings, the termi-

nus of which is attached to a six-carbon (cyclohexane) ring. This arrangement has the appearance of a staircase-like structure — hence the ladderane moniker. A simple modification of the terminal cyclohexane ring results in the other proposed lipid structure, which consists of five cyclobutane rings arranged in a ladder-like array. Further, Damsté *et al.* show that the unique structure, biophysical properties and intracellular location of the lipids are likely to be essential to the metabolic function of the bacteria concerned.

To appreciate the significance of the ladderane lipids, a little information about the microbes containing them is helpful. The ladderane-containing bacteria are distantly related to other microbes belonging to the order Planctomycetales². These bacteria have a highly unusual physiology, in that they live by consuming ammonia in the absence of oxygen. As a consequence of ammonia oxidation, nitrite (NO₂[−]) is reduced, nitrogen gas is generated, and carbon dioxide is converted (‘fixed’) into organic carbon^{3–6}. The overall process, referred to as ‘anammox’, is the central energy-generating pathway for these microbes:



The biological feasibility of this process was predicted decades ago based on thermodynamic considerations⁷. But only recently was it actually shown to occur, in a wastewater treatment plant, by Keunen and colleagues⁸. Anammox is of great practical interest, given the need to remove nitrogen

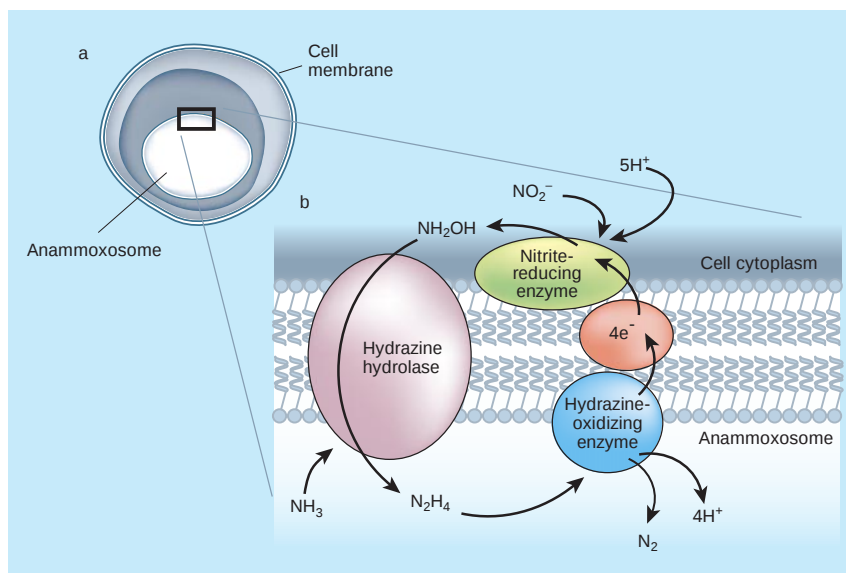


Figure 1 Bacteria with a difference. a, A simplified depiction of the anammox microbe, showing the anammoxosome. This is the organelle-like structure in which the energy-generating process involving the combination of ammonia with nitrite takes place. b, The anammoxosome membrane, which consists of the ladderane lipid bilayer identified by Damsté *et al.*¹, and the anammox reaction pathway. Intermediates in the cycle are hydrazine (N₂H₄) and hydroxylamine (NH₂OH), which are highly toxic. The dense, impermeable membrane may serve to contain them. (Adapted from refs 1 and 6.)

from waste water, and the anammox bacteria perform this job quite admirably. Although they have not yet been isolated in pure culture, the microbes concerned² and the catalytic pathway^{3–6} of the anammox process have been identified, largely through the efforts of Keunen, Jetten and colleagues at the University of Nijmegen. The specifics of the anammox pathway are quite remarkable, and especially startling (Fig. 1) are the highly toxic and reactive intermediates that are formed — hydroxylamine and hydrazine, the second of which finds a very different use as rocket fuel.

The location, structure and biophysical properties of the ladderanes suggest that they have a specific function in anammox bacteria. The enzymes that drive the process are localized in a special intracellular compartment, the anammoxosome (Fig. 1)^{6,9}. This is the same place in which Damsté *et al.* find the ladderanes. Presumably, the rigid structure and dense packing of the ladderane membranes help to contain the noxious anammox intermediates. This would be analogous to the way that eukaryotic organelles called lysosomes compartmentalize digestive enzymes, protecting the rest of the cell from damage.

To test this hypothesis, Damsté *et al.*¹ examined the permeability of the ladderane membrane. Unlike other membrane compartments in the cell, the anammoxosomes were completely impervious to a variety of agents. So the unique structure of the ladderanes does seem to play a role in compartmentalizing the anammox intermediates. In evolutionary terms, one might ponder which came first: the ladderanes, or the anammox pathway? Now that we know what to look for, a search for wider distribution of these lipids may shed light on the question.

“The cell consists of numerous half-living chemical molecules suspended in water and enclosed in an oily film. When the whole sea was a vast chemical laboratory the conditions for the formation of such films must have been relatively favourable.” Thus J. B. S. Haldane¹⁰ imagined the early evolution of one of the most defining structures of life, the cell membrane. Given current theories about those promiscuous times, when lateral genetic transfer is thought to have exceeded vertical transmission¹¹, the invention of a defined cell barrier and the emergence of the cell as ‘self’ was certainly a turning point in the evolution of cellular life. The variety of membrane lipid structures existing in those early times could well have been quite diverse. But innovations in membrane lipid structure as dramatic as that described by Damsté *et al.* appear to be generated rarely today.

These new observations¹ will certainly help in understanding the physiological processes that occur in anammox bacteria, and the mechanisms of subcellular compart-

mentalization in prokaryotes. They may well also provide insight into evolution and innovation in membrane lipid structures in general.

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Evolution

The good, the bad and the lonely

Franziska Michor and Martin A. Nowak

In game theory, ‘loners’ who choose not to participate in fact promote cooperation between players. The dynamics of the game show phase transitions and complex phenomena reminiscent of statistical physics.

Hungarian-born genius John von Neumann, in a rare moment of humility, confessed that he considered himself a failure: according to his own ranking, he was only the third-best mathematician of the time. Yet von Neumann not only built one of the first computers and achieved breakthroughs in fields as diverse as operator theory, mathematical logic and hydrodynamics, he also created several fields of mathematics from scratch. Two of these fields, game theory¹ and cellular automata², are merging in the study of evolutionary dynamics³. Earlier this year, Hauert *et al.*⁴ pushed this development further by investigating the effects of voluntary participation in ‘public-goods games’ — players can choose whether or not to join in a game (or interaction) that may benefit them. That work is now extended by Szabó and Hauert⁵, writing in *Physical Review*

Letters, to a large interacting community of players on spatial grids.

The quest for cooperation is as old as evolution itself. In the *Origin of Species*, Darwin noted that natural selection cannot directly promote altruistic acts where individuals reduce their own competitive ability but increase that of others. Yet cooperation is abundant in nature. The standard explanations that have been developed for this include kin selection, group selection and reciprocity^{6–11}.

The essence of cooperation is captured by the public-goods game. Each individual of a group can decide whether or not to invest some money in a common pool. The common pool is increased by some amount and then equally distributed among all group members regardless of whether or not they made a contribution. The optimum outcome

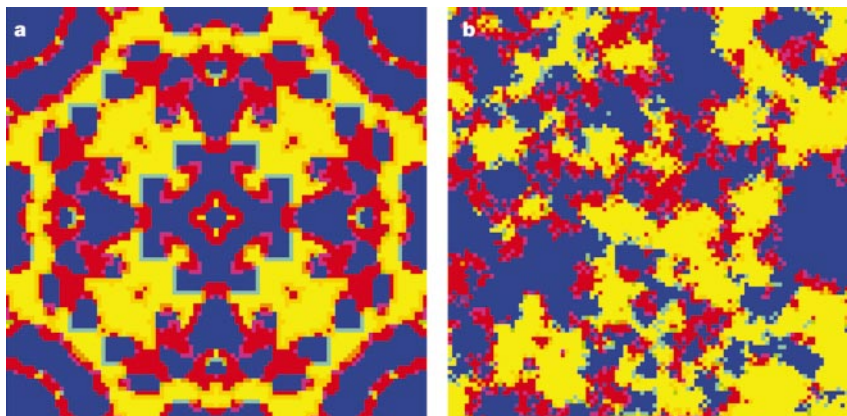


Figure 1 Evolutionary kaleidoscope. a, Players in a public-goods game are represented as cells distributed spatially on a grid. Cooperators are shown in blue, defectors in red and loners in yellow. Players may change their strategy in each round of the game. For this simulation, all strategy updates are synchronous. Intermediate colours indicate players who have updated their strategies in the last round of the game. b, Another simulation shows the effects of asynchronous strategy updating and small amounts of noise in the system. In both cases, there is coexistence between cooperators, defectors and loners. Szabó and Hauert⁵ show that the dynamics of such systems have much in common with models in statistical physics.

for the group occurs if everybody cooperates. But the temptation is to 'free ride': those who don't contribute (defectors) always get a higher pay-off than cooperators who do contribute. If everyone defects, however, no one will enjoy the public goods. Self-interest is self-defeating! This social dilemma threatens the success of public enterprises such as social security, the conservation of environmental resources or group defence against external threats. Notice that the well-known 'prisoner's dilemma' is a public-goods game for groups of two people.

Hauert *et al.*⁴ proposed a new and surprisingly simple mechanism to promote cooperation. Rather than allowing cooperation or defection as the only strategic alternatives, they added a third option — 'loners', who do not participate in the game but instead receive a small, independent pay-off corresponding to a modest income from some self-sufficient occupation. Those who choose to participate in the public-goods game must forgo the loner's pay-off. Equivalently, one could regard the pay-off as the avoidance of a fixed cost for participating in the public-goods game.

In this game, defectors dominate cooperators, as they do in a game without loners; but loners dominate defectors, because in a world of defectors, the public-goods game yields nothing, and loners avoid the cost of participating. Cooperators, on the other hand, dominate loners, because the public-goods game pays in the absence of defectors. The circle is closed. The outcome depends on the details of the underlying evolutionary dynamics, but there are oscillations in the frequencies of the three strategies. For example, if players always adopt the strategy providing a higher pay-off, then the oscillations of the frequencies of all three strategies are stable and robust. The updating mechanism of best-reply dynamics — switching to the best strategy, given the composition of the population at a certain point in time — leads, through damped oscillations, to a stable equilibrium among all three strategies. Hence, the social dilemma is relaxed: instead of defectors winning the world, there is coexistence among cooperators, defectors and loners.

Voluntary participation, though, is only a 'Red Queen' mechanism for cooperation. The Red Queen in Lewis Carroll's *Alice's Adventures in Wonderland* always has to run to stay on the same spot. Similarly, the oscillations among cooperators, defectors and loners lead to average pay-offs that are no larger than having no public-goods game at all. Volunteering does not lead to the fixation of cooperators, but it prevents the fixation of defectors in the population. It needs other mechanisms, such as punishment^{12,13}, to achieve a stable regime of all-out cooperation. Interestingly, however, optional participation does not require individual recognition.

In spatially extended games, as investigated by Szabó and Hauert⁵, voluntary participation is even more successful in promoting cooperation than in well-mixed populations. In the computer simulation, players are represented by cells on a spatial grid. The size of the neighbourhood determines the number of players that interact in the public-goods game. Players with a higher pay-off have an increased probability of being imitated by their neighbours. Hence, successful strategies spread locally. Clusters of cooperators do better than clusters of defectors, but isolated defectors flourish if surrounded by cooperators. In spatial versions without volunteering, there is coexistence between cooperators and defectors for a restricted range of parameters. Adding loners greatly increases the range of parameters allowing coexistence. Hence, optional participation enables the survival of cooperation under circumstances that would normally favour the dominance of defectors.

The intriguing spatiotemporal patterns show cyclic, complex behaviour and coexistence of all three strategies (Fig. 1). Some of the cellular games are closely related to models in statistical mechanics, such as the Ising model for magnetic spins. Spatial games exhibit phase transitions and the full range of complex phenomena observed in the world of cellular automata¹⁴. Hauert has designed a beautiful web page (www.univie.ac.at/virtualabs) where visitors can run simulations for their own choice of parameters and initial conditions. Sometimes, extreme parameter values or small system sizes lead to the extinction of strategies; for example, extinction of defectors in small societies leads to homogeneous populations of cooperators.

At the dawn of life on Earth (or else-

where), replicating molecules had to cooperate to take the first step towards increasing complexity and stability of molecular and later cellular ecosystems. Multicellularity requires cooperation among cells. Cooperation is common in animal societies, but it is often confined to interactions among related individuals. Large-scale cooperation among unrelated individuals seems to be a particularly human trait. Of course, cooperation is always threatened by defection; oscillations between 'war and peace' have been a recurring theme in the cooperation literature. The new work shows that it is optional, rather than compulsory, interactions that promote cooperation. ■

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Immunology

Catch us if you can

Wayne M. Yokoyama

Tumours have ways of evading the body's immune system. A surprising example involves a mechanism that at first sight would seem to have the opposite effect and improve immune responsiveness.

How can tumours develop when the immune system should instead attack and destroy them? Especially difficult to understand is the situation in which tumour cells display surface molecules that should identify them as abnormal, and so should specifically activate immune cells. This makes little sense from the tumour's point of view. These molecules should flag the tumour cells for destruction, unless they somehow give the tumour an advantage — a situation that should remind those of a certain age of the Dave Clark Five, and the

immortal lines of one of this 1960s band's songs: "Here they come again, Catch us if you can." On page 734 of this issue, Groh *et al.*¹ offer insight into this puzzle. They describe a mechanism of tumour evasion that may also apply to the impairment of other immune defence systems, and which may well have clinical potential.

Immune 'effector' cells express surface receptors that are involved in activating the cell by transmitting signals to the cell interior². In the case of specialized immune cells termed cytotoxic T lymphocytes, or CTLs,

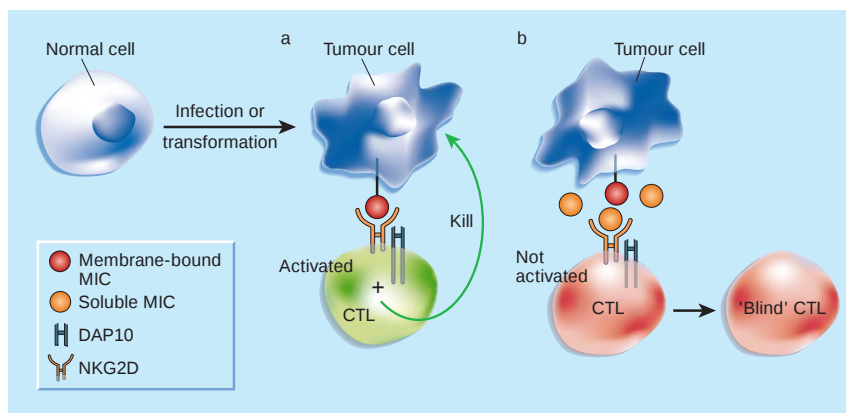


Figure 1 Tumour evasion of the immune system. a, NKG2D ligands such as MIC are often induced in cells by 'stress' — viral infection, for instance, or transformation of the cell into a tumour cell. The result should be that the cell becomes more susceptible to killing by cytotoxic T lymphocytes (CTLs): NKG2D probably works as a receptor that co-stimulates the T-cell receptor (not shown) through the DAP10 signalling chain. b, As Groh *et al.*¹ demonstrate, there are soluble forms of MIC circulating in the blood of patients with MIC-expressing tumours which can affect NKG2D function by blocking recognition of membrane-bound MIC by CTLs and downregulating NKG2D expression. The result is that the CTL effectively becomes blind to the presence of the tumour.

such signals may result in the killing of an adjacent target cell, a process called cytotoxicity². For signalling to occur, the target or tumour cell must display molecules or ligands on its surface that are specifically recognized by the effector cell's activation receptors.

Cytotoxic T lymphocytes possess exquisitely specific activation receptors — T-cell receptors — that initiate signalling². But CTL activation also requires other, co-stimulatory, receptors; otherwise, only partial activation or sometimes immune paralysis occurs. The recently characterized NKG2D receptor takes on this function when it is coupled to a signalling chain known as DAP10 (Fig. 1)³. Many ligands for NKG2D — MICA and MICB (collectively termed MIC) in humans, and RAE1 and H60 molecules in rodents — are induced by stress and do not occur in large amounts under normal circumstances^{4,5}. For example, virus infection can result in the production of MIC, thereby increasing the susceptibility of infected cells to NKG2D-dependent killing by CTLs⁶. Although there are other NKG2D ligands that may be present in normal tissues, they are less well understood⁷. However, we do know that many tumours display stress-induced NKG2D ligands, and that the ligand-expressing tumours are more susceptible to NKG2D-dependent cytotoxicity by CTLs and related natural killer cells than are tumours that lack these ligands. Furthermore, the transfer of genes for NKG2D ligands into tumour cells that do not express these molecules results in enhanced killing.

A peculiar situation thus exists in the many malignancies — especially those of epithelial tissues (breast, ovary, colon, lung and prostate) — whose cells express MIC on their surface *in vitro*, often at high levels, and which therefore show increased susceptibility

to cytotoxicity (Fig. 1a). This is seen even in freshly isolated tumour cells, so that artefacts of *in vitro* cell culture cannot explain MIC expression¹. And freshly removed, carcinogen-induced mouse tumours express RAE1 and H60 (ref. 8). So why would tumours display molecules that increase their sensitivity to the immune system?

Groh *et al.*¹ have come up with an answer. They show that cancer patients whose tumour cells express surface MIC (MIC⁺) also have soluble forms of MIC (sMIC) in their blood (Fig. 1b). Blood-serum extracts from these patients decrease NKG2D expression and function, reactions that are also seen when normal lymphocytes are exposed to recombinant sMIC or to MIC⁺ or RAE1⁺ tumours themselves^{1,9}. Also, NKG2D expression on circulating lymphocytes is abnormal in patients with MIC⁺ tumours, suggesting that the function of most lymphocytes may be affected. Obviously, the way in which sMIC expression correlates with clinical outcome, and its potential for diagnosing and monitoring patients, require further investigation. Whether these observations on sMIC extend to other NKG2D ligands also remains to be seen.

But there is another interesting aspect of these findings. Because NKG2D may be involved in host defence against many pathogens^{6,7,10–12}, persistent overall down-regulation of NKG2D may provide another explanation for cancer patients' predisposition to infections. So the findings of Groh *et al.* might explain not only tumour evasion but also other aspects of immune suppression in cancer patients. Furthermore, these findings suggest that a similar evasion strategy might exist for other receptors and ligands in the immune system.

Soluble MIC affects NKG2D function in at

least two ways — by acting as a competitive mimic that blocks recognition of membrane-bound ligand, and as a suppressor that down-regulates NKG2D expression (Fig. 1b)¹. The mechanism of this second activity is unclear, but is reminiscent of the ligand-stimulated internalization of other lymphocyte-activation receptors by soluble ligands⁷. In either case, it is notable that NKG2D has very high affinities and structural plasticity for its ligands^{13,14}. This may partly explain why sMIC has a marked effect on NKG2D even when it is virtually undetectable by immunoassay. Regardless of this, sMIC could be used as a therapeutic agent to inhibit immune cells when the immune system is overactive, as occurs in autoimmune diseases. These include conditions where MIC expression is enhanced, such as inflammatory bowel disease¹², and perhaps even disorders where MIC itself is not involved — NKG2D is widely expressed on large numbers of immune cells and has many other ligands.

How does sMIC arise? A clue comes from the association of sMIC with MIC⁺ tumours, from which it seems that surface-bound MIC may give rise to sMIC. This could occur by specific enzymatic cleavage, as is seen in the processing of soluble forms of members of the tumour-necrosis-factor family². On the other hand, alternatively spliced forms of MIC RNA transcripts for the soluble forms might be produced at the same time as transcripts for the membrane-bound forms. Whatever the answer, this question should spawn research into potential pharmaceutical targets. If the first mechanism is involved, blockade of the enzymatic event could prevent sMIC from being produced, and possibly increase production of the uncleaved membrane-bound forms that flag tumours for destruction by NKG2D-activated immune cells. We therefore have a new hint as to how to catch the tumour cells that have long eluded both the immune system and immunologists.

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Materials chemistry

Liquid crystals stack up

Carsten Tschierske

Take a spherical carbon 'buckyball', feather it with rod-like molecules, and the result is a distinctive shuttlecock shape that can easily be stacked into columns. Liquid-crystal phases thus formed should have unusual properties.

Liquid crystals (LCs) are materials that can flow like fluids, but also have some of the regularity and direction-dependent properties of crystals. This combination of order and mobility enables these systems to respond to external stimuli, such as an applied electric field, and change their organization. For this reason, they are ideal components for flat-panel devices in computers, cellular phones and televisions, but numerous future applications in nonlinear optics, molecular electronics and molecular photonics are conceivable¹. On page 702 of this issue, Sawamura *et al.*² describe a new type of liquid-crystalline material. They have synthesized molecules that are shaped like a badminton shuttlecock and stack together in a directed manner.

Conventional LC materials are mostly formed by rod-like or disc-like molecules that have flexible chains attached to rigid cores (Fig. 1a, b). In most cases, rod-like molecules organize themselves into layers (known as smectic LC phases), whereas disc-shaped molecules form columns (columnar LC phases)¹. The chains are fluid and provide the necessary mobility which, in particular temperature ranges, inhibits the formation of solid crystals.

If the symmetry of such molecules is reduced, different LC phases can arise. For example, the introduction of a bend of about 110–140° at the centres of rod-like molecules leads to 'banana-shaped' molecules (Fig. 1c) which can organize to provide LC phases not found in linear rod-like molecules³. When such bent-core molecules organize in layers, their specific shape gives rise to a directionality of alignment, causing a polar order in each of the layers. This polar order and the reduced phase symmetry yield extremely interesting phenomena, such as the spontaneous formation of chiral and helical superstructures, and ferroelectric or antiferroelectric properties^{3–6}. In ferroelectric phases, the polar directions of neighbouring layers point in the same direction, leading to macroscopic polar order, whereas in antiferroelectric phases the polar direction in adjacent layers is opposite, so that the polar moments are cancelled (Fig. 1c). Such ferroelectric and antiferroelectric phases are potentially useful, because they can be switched by electric fields between distinct states.

Several attempts have been made to realize polar order in columnar LC phases^{7,8} —

which could be expected if the flat shapes of disc-like molecules were bent into bowl-like or hollow cone shapes (Fig. 1d). Sawamura *et al.*² provide a new concept for the design of hollow cone molecules, using the buckminsterfullerene molecule (C_{60})⁹ as the central unit. C_{60} has special redox and photophysical properties and so there is great interest in incorporating it into LC materials¹⁰. Sawamura *et al.* attached five rod-like aromatic units, each bearing two flexible non-aromatic chains, to one side of the C_{60} molecule to form a cone-shaped molecule resembling a shuttlecock (Fig. 1e).

The C_{60} apex of each of these molecules

fits perfectly into the cavity of a neighbouring molecule. As a result, the molecules prefer to organize head-to-tail in columns, and the columns themselves organize parallel to each other in a regular manner, forming a hexagonal two-dimensional lattice. The flexible chains at the periphery fill the space between the columns and provide the necessary mobility for LC formation. But, in contrast to polar layers of banana-shaped molecules which can easily adopt a macroscopically non-polar antiferroelectric arrangement by alternation of the polar direction of adjacent layers (Fig. 1c), the organization of polar columns in a hexagonal two-dimensional lattice is more complex. There are many ways for the polar columns to be arranged (examples are shown in Fig. 2, overleaf), but it is not possible to arrange them in such a manner that each column only has neighbours of opposite polar direction. Hence, for these systems many new polar LC phases could be expected.

For such polar LC phases to be exploited

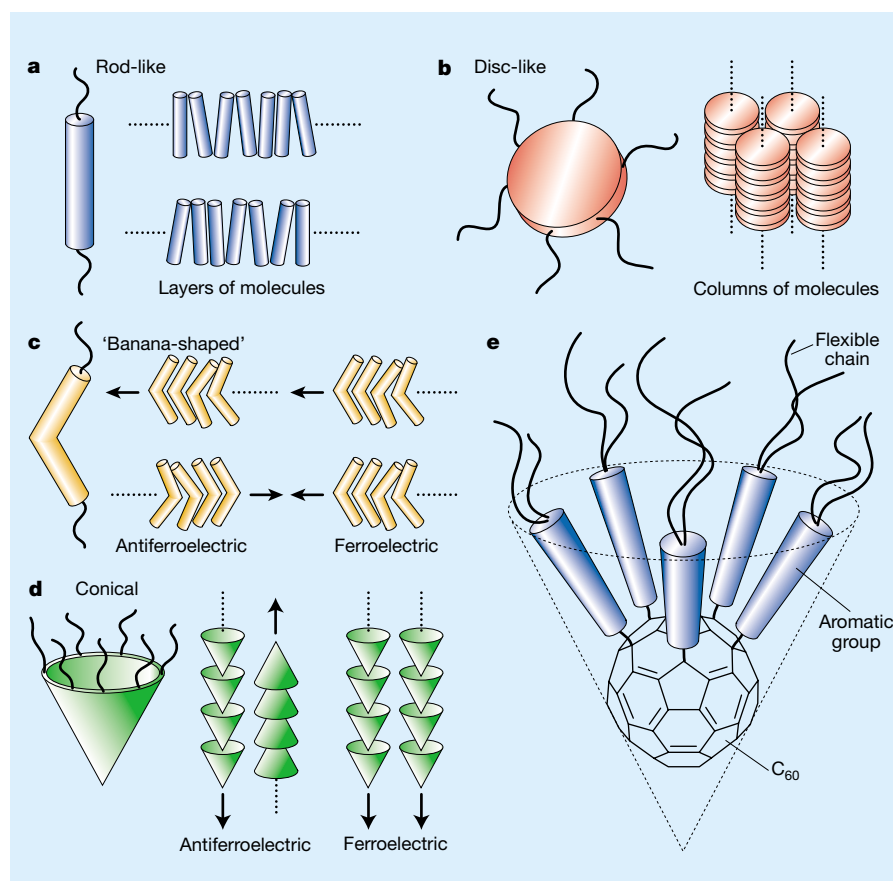


Figure 1 Liquid-crystal phases. a, Rod-like molecules organize themselves into layers, whereas, b, disc-like molecules form columns that can be arranged parallel to each other in a two-dimensional lattice. c, A bend introduced in the rigid core leads to 'banana-shaped' molecules. The rotation of these molecules around their long axis is restricted and they adopt a directed order within the layers. Depending on the bending direction in adjacent layers, either antiferroelectric or ferroelectric smectic phases may result. d, Molecules with a conical shape can lead to a polar order within columns. The polar direction of neighbouring columns may be parallel or antiparallel. e, Sawamura *et al.*² have made a 'shuttlecock-shaped' molecule based on the C_{60} molecule, whose distinctive shape leads to directed organization in columns.

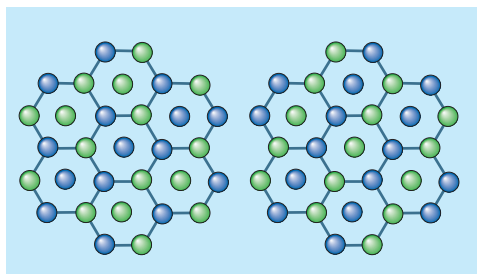


Figure 2 Examples of hexagonal lattices with polar columns (coloured green and blue for the two orientations). Note that it is not possible to arrange the columns in such a manner that each polar column only has neighbours of the opposite polar direction.

in switching processes, the molecules must be mobile enough to respond quickly to external stimuli. High viscosity is a general problem of columnar phases, but it can be much reduced by adding solvents that dissolve around and between the flexible chains^{11,12}. As well as reducing viscosity, this can lead to a change of the organization in the LC phase: in the case reported by Sawamura *et al.*², the hexagonal columnar LC phase was replaced by a columnar 'nematic' phase at higher solvent concentrations and temperatures. In this phase, the long-range hexagonal lattice is lost and the columns are only on average aligned parallel to each other. Nevertheless, these nematic phases

may also have special properties resulting from the polar order within the columns¹³.

Many of the advances in LC research have been stimulated by fresh designs of molecules that form new LC phases. In their 'shuttlecock' molecules, Sawamura *et al.*² have undoubtedly provided a new design principle for research teams to play with. ■

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Neurobiology

Neurotrophin channels excitement

Yves-Alain Barde

Sodium-ion channels usually open in response to a voltage change across the membrane in which they sit. But, surprisingly, a growth factor secreted by neurons also rapidly triggers the opening of a specific sodium channel.

Ion channels are remarkable proteins that regulate the transfer of ions across the lipid bilayers that make up cellular membranes. These proteins are required for a variety of physiological processes; for instance, a group of cation-selective channels is responsible for conducting action potentials along nerve cells, allowing them to transfer information from one place to another. In vertebrates, the ions that are typically responsible for the propagation of action potentials are sodium ions. These are temporarily allowed to enter neurons through channels in the cell membrane that open as a result of a transmembrane voltage change. Surprisingly, however, Konnerth and colleagues¹ now report (page 687 of this issue) that a brain protein secreted by neurons is involved in opening a particular sodium channel — one already known to occur in sensory neurons of the peripheral nervous system.

The secreted protein in question is a growth factor called brain-derived neurotrophic factor (BDNF), and the story began three years ago when Konnerth and colleagues² found that BDNF causes membrane depolarization within milliseconds. (Membrane depolarization refers to a shift in the potential difference across the membrane that, beyond a certain threshold, can trigger action potentials.) This was a remarkable finding because, until then, only neurotransmitters had been found to have such a rapid effect on the membrane potential of neurons. Typical neurotransmitters are low-molecular-weight compounds such as acetylcholine and the amino acids glutamate and glycine. They act on a millisecond timescale because they bind to receptors that are also ion channels, causing them to open briefly.

By contrast, BDNF is a much larger mol-

ecule, consisting of two identical chains of about 100 amino acids each, and neither of the two known receptors to which it binds is an ion channel. In fact, one BDNF receptor is an enzyme, the transmembrane tyrosine kinase TrkB, which, with a certain delay, mediates some of the typical biological activities of BDNF — such as the prevention of neuronal death³ (Fig. 1, overleaf). Yet Konnerth and colleagues clearly showed that BDNF causes rapid membrane depolarization, and that briefly exposing TrkB-expressing neurons to puffs of BDNF triggers trains of action potentials². Other neurotrophins that have different receptor specificities failed to do this.

Although surprising, these results helped to explain previous observations indicating that BDNF modulates synaptic transmission⁴ (neurons typically communicate at synapses, which are specialized junctions). One particularly famous example of such modulation is the long-term potentiation (LTP) of synaptic transmission. This is a widely used cellular model of memory, and numerous studies have shown that in the hippocampus — a brain structure crucial to memory formation in rodents and humans — BDNF plays a role in LTP that is essentially as significant as that of glutamate⁴. The observed BDNF-induced membrane depolarization triggered an increase in the levels of Ca²⁺ ions in the protrusions (spines) of post-synaptic neurons — those on the receiving side of the synapse — and immediately induced LTP when paired with a burst of synaptic stimulation⁵.

In an impressive reconstitution experiment, Konnerth and colleagues¹ now show which molecules are needed for BDNF to rapidly depolarize neurons. Briefly, when the authors created fibroblast cells containing a particular subtype of Na⁺ channel, Na_v1.9, together with the BDNF receptor TrkB, they found that BDNF caused an inward flow of Na⁺ ions, and hence membrane depolarization, within milliseconds. This is simply too rapid to be attributed to the enzymatic activity of TrkB. Moreover, in these experiments, Na_v1.9 could not be activated by voltage changes alone, although the related channel Na_v1.7 could. So the explanation must be that binding to BDNF produces a conformational change in TrkB that is transferred without delay to Na_v1.9, such that it briefly becomes permeable to Na⁺ ions. Finally, Konnerth and colleagues found that, in hippocampal neurons, the targeted elimination of Na_v1.9 blocks BDNF-induced depolarization. This implies that these neurons, at least, contain Na_v1.9 and require it for the BDNF-mediated influx of Na⁺ ions.

The unexpected conclusion that a secreted growth factor can modulate the opening (gating) of an ion channel on the same

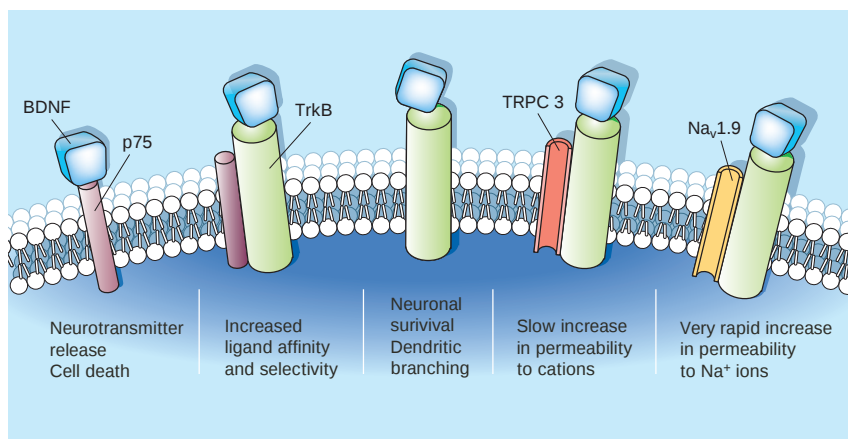


Figure 1 Brain-derived neurotrophic factor (BDNF) is a versatile protein. There are two receptors for BDNF on the surface of neurons: the neurotrophin receptor p75, which binds all neurotrophins, and TrkB. Each receptor alone binds BDNF with nanomolar affinity, but when they associate, the affinity and selectivity of TrkB for BDNF increase. TrkB can also associate with two different ion channels. TRPC3 is a non-selective cation channel that needs to be phosphorylated by TrkB to open; this is a slow process (acting in the range of minutes). Na_v1.9 is selective for Na⁺ ions and, as Konnerth and colleagues¹ show, opens within milliseconds following the binding of BDNF to TrkB. The figure also shows some of the functional outcomes of the activation of the BDNF receptors.

timescale as a voltage change raises several questions. The Na_v1.9 channel is best known for its presence in small sensory neurons of the dorsal-root and trigeminal ganglia⁶, where, together with Na_v1.8, it may play a key part in the physiology of neurons involved in pain perception^{6,7}. The expression of Na_v1.9 is regulated by another growth factor, glial-cell-derived neurotrophic factor (GDNF). These sensory neurons do not express TrkB, so it will be interesting to see whether one component of the GDNF receptor, the tyrosine kinase Ret, is also associated with Na_v1.9, and whether GDNF causes rapid changes in membrane potential. If so, this could have implications for our understanding of pain sensing.

In a similar vein, most Na_v1.8-expressing and many Na_v1.9-expressing neurons also express TrkA⁷—the tyrosine kinase receptor for nerve growth factor (NGF). NGF has been associated with pain sensing in many ways⁸, and one wonders whether it may trigger the rapid opening of Na_v1.8 or Na_v1.9 via TrkA.

Another question is whether the BDNF-induced, TrkB-mediated depolarization seen in all neurons of the central nervous system (CNS) tested so far can be attributed to Na_v1.9. Although Konnerth and colleagues' latest report¹ indicates that Na_v1.9 is expressed by hippocampal CNS neurons, the levels of expression are probably much lower than those in sensory neurons⁶. Finally, it has not yet been demonstrated biochemically that TrkB associates directly with Na_v1.9, although TrkB does associate with other transmembrane proteins^{3,9}, such as the neurotrophin receptor p75 and the nonspecific cation channel TRPC3 (Fig. 1).

Intriguingly, the binding of BDNF to TrkB also increased the permeability of the TRPC3 channel⁹, although this required the enzymatic activity of TrkB and was considerably slower (minutes instead of milliseconds).

Thus, one of the last remaining distinctions between the physiological properties of neurotrophins and neurotransmitters seems to have disappeared. Work on neurotransmitters may become a source of inspiration for researchers interested in the neurophysiology of neurotrophic factors, and one of the next questions may be how the effects of BDNF on ion channels are rapidly inactivated. Mechanisms involving receptor internalization might be too slow to eliminate this poorly diffusible protein, which is also fairly resistant to protein-degrading enzymes. But BDNF-induced desensitization, as occurs with neurotransmitter receptors, may be an attractive possibility to explore.

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100 YEARS AGO

The question I wish to bring to your notice to-day is an old one: if two events happen simultaneously or one follows the other at a short interval of time, does this give us any reason to suppose that these two events are connected with each other, both being due to the same cause, or one being the cause of the other? Everyone admits that the simple concurrence of events proves nothing, but if the same combination recurs sufficiently often we may reasonably conclude that there is a real connection. The question to be decided in each case is what is "sufficient" and what is "reasonable". Here we must draw a distinction between experiment and observation. ... The cause of the difference lies in the fact that in an experiment we can control to a great extent all the circumstances on which the result depends, and we are generally right in assuming that an experiment which gives a certain result on three successive days will do so always. But even this sometimes depends on the fact that the apparatus is not disturbed, and that the housemaid has not come in to dust the room. Here lies the difference.

From *Nature* 16 October 1902.

50 YEARS AGO

Our present political systems are likely to be modified. Democracy lacks survival value. The state of parasitism on the community engendered by modern social conditions is impermanent because in the end the parasite destroys its host and then itself perishes: the process is likely to be hastened by the concomitant reduction of the more intelligent, these being now driven to have few or no children. It will be the non-parasitic communities that will survive, and those that multiply most will dominate the earth by sheer numbers. For this reason the author thinks that world agreement to control population could never be attained: an aggressor nation or bloc would never permit their people to restrict their numbers, and other nations that wished to survive would have to follow suit. Government would possibly be by some "hero" who has sufficient sense to adapt himself to a society of dense population. Here intellect will count, but morality may not, since "in a highly competitive world, the sinner has many advantages over the saint".

From *Nature* 18 October 1952.

An onion enzyme that makes the eyes water

A flavoursome, user-friendly bulb would give no cause for tears when chopped up.

The irritating lachrymatory factor that is released by onions when they are chopped up has been presumed to be produced spontaneously following the action of the enzyme alliinase, which operates in the biochemical pathway that produces the compounds responsible for the onion's characteristic flavour¹⁻⁴. Here we show that this factor is not formed as a by-product of this reaction, but that it is specifically synthesized by a previously undiscovered enzyme, lachrymatory-factor synthase. It may be possible to develop a non-lachrymatory onion that still retains its characteristic flavour and high nutritional value by downregulating the activity of this synthase enzyme.

Previous studies¹⁻⁴ indicated that alliinase from any source was the only enzyme needed to produce lachrymatory factor (propanthial S-oxide) from 1-propenyl-L-cysteine sulfoxide (PRENCISO), an important substrate in onion (*Allium cepa*) (Fig. 1a). The reactions from the intermediate sulphenic acid to propanthial S-oxide and thiosulphinates were presumed to be spontaneous because sulphenic acid is very unstable and has never been isolated.

When we added crude extract of alliinase from garlic (*A. sativum*) to PRENCISO, however, lachrymatory factor was not produced at all, and the yield of thiosulphinates increased. Because a crude preparation of alliinase from onion added to PRENCISO could generate the factor, we investigated whether some unknown component (possibly another enzyme) in the preparation could be involved in its formation.

The fraction with lachrymatory-factor-

forming activity could be completely separated from the alliinase activity by passing the crude onion alliinase preparation through a hydroxyapatite column. Further purification of this fraction gave three distinct proteins, whose amino-terminal sequences we determined.

We used the RACE (rapid amplification of complementary DNA ends) technique with degenerate gene-specific primers deduced from one of the amino-terminal sequences to obtain a complete cDNA sequence. The full-length cDNA (GenBank accession no. AB089203) consisted of 737 base pairs, with a predicted gene product of 169 amino acids.

As all of the amino-terminal sequences determined for the three proteins matched the predicted open reading frame of the gene, we assumed that these three proteins were the products of a single gene. DNA-database searches revealed that the gene encoded a new enzyme, which we named lachrymatory-factor synthase.

When we expressed the lachrymatory-factor synthase gene in *Escherichia coli*, the resulting recombinant protein exhibited the expected enzymatic activity. The enzyme showed high substrate specificity, producing lachrymatory factor from only *trans*-PRENCISO, which is the naturally occurring form in onion (Fig. 2).

Lachrymatory factor was detected only when all three components, namely purified alliinase, PRENCISO and lachrymatory-factor synthase, were present in the reaction mixture (Fig. 1b). Omission of the synthase from the reaction mixture resulted in an increased yield of thiosulphinates,



Figure 2 Don't cry for me: inhibiting the biosynthesis of lachrymatory factor could give rise to a no-more-tears formula for onions.

the condensation product of 1-propenylsulphenic acid.

These results indicate that it might be possible to develop a non-lachrymatory onion by suppressing the lachrymatory-factor synthase gene while increasing the yield of thiosulphinates. Thiosulphinates is responsible for the flavour of fresh onion, and is converted to compounds reported to exert hypolipodaemic⁵ and antiplatelet-aggregation effects^{6,7}. Although down-regulating alliinase itself would also lead to a non-lachrymatory onion, its flavour and nutritional value might be compromised.

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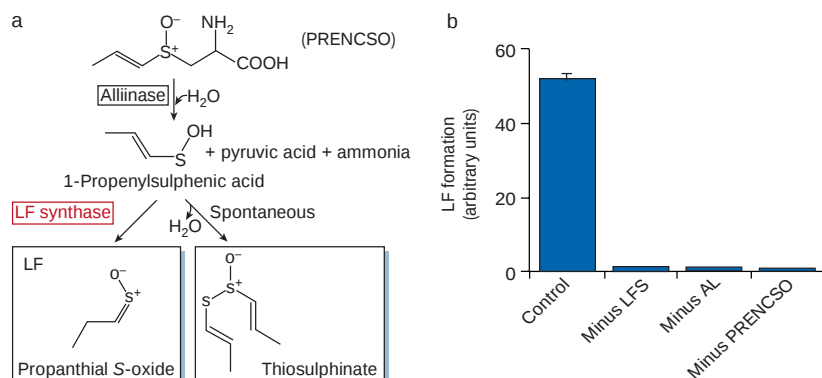


Figure 1 Obligatory involvement of the enzyme lachrymatory-factor synthase in the production of lachrymatory factor (LF) in onion. **a**, The chemical reactions that occur when onion bulbs are cut. Propanthial S-oxide (LF, responsible for stimulating tears) and thiosulphinates (which determines the onion's flavour and leads to the production of biologically active compounds in the onion) are generated; the step involving the newly discovered enzyme LF synthase is indicated. Previously, lachrymatory factor was believed to be formed spontaneously following the action of alliinase after cutting. **b**, LF is formed only in the presence of its synthase (LFS), alliinase (AL) and PRENCISO (control, left). If any one of the three components in the reaction mixture is omitted, no LF is detectable (right three bars). The LF produced was separated on an HPLC column, and the amount was determined from the flow-through peak area (arbitrary units).

The ventilatory response to hypoxia

Respiratory physiologists traditionally attribute the increased ventilatory response to hypoxia to increased discharge by the carotid-body chemoreceptor, which is transmitted by sensory processes to neurons in the medullary nucleus of the solitary tract¹. However, Lipton *et al.* propose a radically new model² in which hypoxia causes haemoglobin to release molecules derived from nitric oxide, which then increase ventilation by directly stimulating solitary-tract neurons. Despite the apparent feasibility of this model^{3–5}, we show here that the observations of Lipton *et al.*² do not invalidate the classic carotid-body-mediated explanation of the hypoxic ventilatory response. We thus question the justification for a new model to account for hypoxia's effect on breathing.

Lipton and colleagues' proposal that molecules derived from nitric oxide, called S-nitrosothiols (SNOs), duplicate the physiological response to hypoxia is based on three observations. First, they observed an equivalent increase in ventilation after separate injections into the rat nucleus tractus solitarius (NTS) of equimolar concentrations of S-nitrosocysteine (L-CSNO), S-nitroso-L-cysteine (L-CSNO) and S-nitrosoglutathione (GSNO). Second, injection of an extract of deoxygenated, but not oxygenated, blood reproduced the effect of these agents. Third, the response to 10% O₂ mirrored that produced by CGSNO, CSNO and GSNO, and the decay characteristics of the recovery of ventilation were identical in each case. No data are presented to support this final claim, but the authors' Figs 2 and 3 reveal widely different time constants of about 120 seconds after hypoxia, 60 s after CGSNO, and 240 s after blood extract.

If SNOs mediate the hypoxic ventilatory response (HVR), the effect of SNOs and hypoxia should disappear in response to blocking any step between the proposed release of SNOs at the NTS and the increased respiratory motor output. Acivicin, which blocks a step involving γ -glutamyl transpeptidase (γ -GT), abolished the ventilatory response to GSNO in rats, but the HVR persisted, as it did in mice genetically deficient in γ -GT (ref. 2). SNOs thus increase ventilation at the NTS through a γ -GT-dependent mechanism, but the HVR is independent of γ -GT; SNOs are therefore not essential for this response.

Lipton *et al.* also propose that SNOs are crucial for the return of ventilation to normal when air replaces hypoxic inspired gas², with SNOs effecting a residual stimulation of ventilation during this recovery phase. This suggestion is based on their findings that

pretreatment with acivicin leads to a marked acceleration of ventilatory decay after hypoxia in rats, and that hypoxic ventilatory recovery is profoundly attenuated in knockout mice that lack γ -GT, which manifests itself as an undershoot in ventilation after 60 s.

We contend that key features of this evidence conflict with earlier work. Ventilation is shown as increasing continuously during 5 min of hypoxia in rats², whereas the typical response is a rapid increase in ventilation, then a decline as hypoxia continues; this well-known biphasic HVR occurs in many species, including humans⁶ and, crucially, mice⁷ and rats⁸. The slow decline in ventilation after a return to air² is also unusual because ventilation falls rapidly on termination of hypoxia in humans⁹, lambs¹⁰ and mice⁷.

We suggest that the 3-litre plethysmograph used by Lipton *et al.*, through which gas passed at 8 litres per min, is likely to have slowed changes in gas concentration². Consistent with this, the reported ramp-like increase in ventilation in response to hypoxia² is identical to that seen in lambs exposed to progressive hypoxia¹⁰, and the 2-min return to baseline ventilation upon returning to air² suggests that hypoxia is persisting.

Furthermore, although the ventilatory undershoot after returning to air is considered to be abnormal², it is in fact well documented^{7,9,10} and has been explained by the development of hypocapnia during the

Gozal *et al.* reply — We do not challenge the 'classic' theory of peripheral-chemoreceptor-mediated HVR. However, this offers no insight into the mediators of the time-domain components of HVR, such as ventilatory short-term potentiation (VSTP)¹. VSTP is critical to respiratory-system stability² and is unrelated to the activity of the peripheral chemoreceptor^{3,4}, so other factors may have a role in HVR^{5,6}. One such factor involves SNO signalling in brainstem neurons. SNOs could be formed by neuronal nitric oxide synthase (NOS, activated by afferents from peripheral chemoreceptors) and by erythrocyte deoxygenation. Indeed, erythrocyte deoxygenation could be signalled to peripheral chemoreceptors through SNO formation.

When concentrations of SNO delivered to the dorsocaudal brainstem match the magnitude of peak HVR, the time constants of VSTP after hypoxia and SNO are similar (our unpublished results). Thus VSTP, a principal component of HVR, is critically dependent on SNO formation and its subsequent activity in brainstem structures. These results are supported by data that demonstrate the NOS-dependency of both VSTP and long-term facilitation following hypoxia⁷.

On the contributions of hypocapnia and ramp-presentation of the hypoxic stimulus to the ventilatory undershoot, following cessation of hypoxic gas administration:

HVR⁹. The duration of hypoxic exposure and the associated fall in blood and tissue CO₂ levels influence whether undershoot occurs⁹ — so without information on blood gases², a chemical-control explanation for the presence or absence of undershoot cannot be discounted.

We suggest that attribution of an important role to SNOs in the HVR is not justified on the basis of Lipton and colleagues' present results, a conclusion that is supported by a wealth of evidence that the response to hypoxaemia is negligible in animals and humans with denervated or resected carotid bodies¹¹.

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isocapnic hypoxia and step presentation of hypoxic stimuli to γ -GT-knockout and wild-type mice confirm the anticipated absence and presence of VSTP, respectively (our unpublished results).

S-nitrosoglutathione may not be the only SNO that signals HVR, because other SNOs are also formed during erythrocyte deoxygenation and NOS activation^{8,9}. We used GSNO as a reporter SNO, but found that other L-isomeric SNOs mimic HVR, as indicated by the large increase in HVR in humans treated with N-acetyl-L-cysteine¹⁰.

Peripheral chemoreceptors are therefore important in initiating the ventilatory response to hypoxia, and SNO signalling is crucial in determining the characteristics of the HVR. It is not necessary to assume that these two features are mutually exclusive.

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Neurotrophin-evoked depolarization requires the sodium channel Na_v1.9

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Brain-derived neurotrophic factor (BDNF) and other neurotrophins are essential for normal brain function. Many types of neurons in the central nervous system are excited by BDNF or neurotrophin-4/5, an action that has recently been implicated in synaptic plasticity. The mechanisms involved in this transmitter-like action of neurotrophins remains unclear. Here, by screening candidate genes with an antisense messenger RNA expression approach and by co-expressing the receptor tyrosine kinase TrkB and various sodium channels, we demonstrate that the tetrodotoxin-insensitive sodium channel Na_v1.9 underlies the neurotrophin-evoked excitation. These results establish the molecular basis of neurotrophin-evoked depolarization and reveal a mechanism of ligand-mediated sodium channel activation.

Mammalian neurotrophins are a family of neurotrophic proteins that includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 and neurotrophin-4/5 (ref. 1). The role of neurotrophins as regulatory factors that mediate differentiation and survival of neurons in the peripheral and central nervous system is well established^{2–4}. The biosynthesis and release of neurotrophins, such as BDNF, is sensitive to electrical activity and synaptic stimulation⁵. There is experimental evidence indicating that activity-dependent release of BDNF may occur pre-synaptically⁶ as well as post-synaptically⁷ in neurons of the central nervous system (CNS). The neurotrophins mediate their actions by two types of receptors; that is, Trk receptor tyrosine kinases and the pan-neurotrophin receptor p75^{NTR}. Each type of Trk is activated by one or several neurotrophins: TrkA by NGF, TrkB by BDNF or neurotrophin-4/5, and TrkC by neurotrophin-3. Similar to other receptor tyrosine kinases, Trk receptors—when bound by their preferred ligands—transphosphorylate tyrosine residues within the cytoplasmic domain and activate a wide range of signalling pathways.

Evidence suggests that neurotrophins participate in activity-dependent neuronal plasticity^{8–10}. Thus, BDNF has been implicated directly in hippocampal long-term potentiation (LTP)—the probably best-established cellular model for learning and memory. In hippocampal CA1 (refs 11, 12) and dentate granule neurons¹³, BDNF is able to induce LTP, and mice that lack the *bdnf* gene have impaired LTP^{14,15}. The conditional knockout of the *TrkB* gene in the forebrain exhibits an impaired learning behaviour¹⁶ and reduced hippocampal LTP^{16,17}. These results support a direct implication of the neurotrophin BDNF–TrkB system in the formation of long-term memory. However, the coupling of BDNF–TrkB signalling to neuronal activity is poorly understood. In neurons of the CNS, BDNF bound to TrkB can activate the non-selective cation channel TRPC3 through the phospholipase C/inositol trisphosphate pathway¹⁸. Another, much faster and distinctly different excitatory action of neurotrophins was reported by ref. 19. In various types of CNS neurons, the study found that low nanomolar levels of BDNF evoke neuronal excitation by activating a Na⁺ current (*I*_{Na}). This BDNF-evoked *I*_{Na} underlying neuronal depolarization was activated within milliseconds and, as a function of the BDNF dose and the time of application, resulted in trains of action potentials¹⁹. One report identified dendritic spines as highly sensitive sites for the BDNF-evoked excitation, and demonstrated that in hippocampal dentate granule cells induction of BDNF-mediated LTP occurs post-synaptically¹³; however, the mechanism underlying BDNF-evoked *I*_{Na} remained unknown. Here we report the identi-

fication of the molecular determinants underlying the BDNF-evoked membrane depolarization. We demonstrate that the ligand BDNF, bound to the receptor tyrosine kinase TrkB, can evoke membrane depolarization through rapid activation of the sodium channel Na_v1.9. Na_v1.9 (ref. 20) (also known as SCN11a/12a and NaN²¹) is a tetrodotoxin (TTX)-insensitive member of the family of voltage-gated sodium channels^{22,23}.

BDNF-evoked *I*_{Na} is highly sensitive to saxitoxin

A pre-requisite for the molecular characterization of BDNF-evoked *I*_{Na} was the identification of a high-affinity antagonist. As reported previously¹⁹, BDNF-evoked *I*_{Na} is insensitive to the common sodium channel antagonist TTX. At a concentration of 50 nM, which causes a complete block of action potential firing (not shown), TTX did not affect BDNF-evoked *I*_{Na} in CA1 pyramidal neurons in rat hippocampal slices (Fig. 1a, b) (19 out of 19 cells). By contrast, BDNF-evoked *I*_{Na} was completely and reversibly blocked by 10 nM saxitoxin (STX) (Fig. 1a, b). Notably, the BDNF-evoked *I*_{Na} sensitivity for STX (half-maximal inhibitory concentration, IC₅₀ = 4.89 nM) is not distinguishable from that found for conventional voltage-gated Na⁺ channels^{24,25}.

We next identified the human neuroblastoma cell line SH-SY5Y as a useful model system for cloning and antisense expression studies. In differentiated SH-SY5Y cells, neuritogenesis and cell survival require BDNF and an upregulation of Trk receptors^{26,27}. We found that SH-SY5Y cells exhibit a BDNF-evoked current that is pharmacologically identical to that recorded in neurons. As in hippocampal CA1 pyramidal neurons (Fig. 1a), BDNF-evoked *I*_{Na} was readily evoked in SH-SY5Y cells (Fig. 1c) that were differentiated for 3–5 days with 10 μM all-*trans* retinoic acid. This BDNF-evoked *I*_{Na} activity was completely and reversibly blocked by 10 nM STX, but was not affected by 50 nM TTX (16 out of 16 cells) (Fig. 1c, d).

Candidate genes underlying BDNF-evoked excitation

To identify the critical molecular determinants involved in neurotrophin-evoked depolarization, we first examined which Trk-receptor subtypes were expressed in SH-SY5Y cells. By using polymerase chain reaction with reverse transcriptase (RT-PCR), we detected TrkB as well as TrkC, but not TrkA, transcripts in SH-SY5Y cells (Fig. 1e). This result is consistent with earlier pharmacological data obtained in hippocampal neurons^{13,19} suggesting that TrkB is the most probable receptor mediating BDNF-evoked *I*_{Na}. We next analysed the expression of voltage-gated sodium channels in SH-

SH-SY5Y cells. For this purpose we devised an RT-PCR approach using degenerate primers to identify multiple sodium channel transcripts from SH-SY5Y mRNA preparations. Primers were deduced from evolutionarily conserved amino acid sequences in the pore regions of sodium channels that are known to belong to the TTX- and STX-binding domains (domain DI-SS2 for upper primers; DII-SS2 for lower primers)^{28,29}. RT-PCR products were analysed by gel electrophoresis (Fig. 1f) and amplification products were identified by Southern blot hybridization using a degenerate, internal oligonucleotide. Finally, positive clones were obtained and analysed by double-strand DNA sequencing. Using this approach we detected transcripts of four members of the voltage-gated sodium channel family in SH-SY5Y cells. Three transcripts were identified as the TTX-sensitive, voltage-gated sodium channels $\text{Na}_v1.2$ (ref. 30), $\text{Na}_v1.3$ (ref. 31) and $\text{Na}_v1.7$ (ref. 32) (also known as SCN2a , SCN3a and NE-Na , respectively). The fourth transcript represented the sodium channel $\text{Na}_v1.9$ (refs 20, 22, 23) (Fig. 1f). In contrast to the other identified members, this sodium channel carries a protein sequence motif typical for TTX-insensitive/STX-sensitive voltage-gated sodium channels in its corresponding protein domains. The

exchange of a tyrosine (Y) or a phenylalanine (F) residue to a cysteine (C) or a serine (S) residue in the domain DI-SS2 of sodium channels leads to a more than 200-fold decrease in TTX affinity, whereas the sensitivity to STX remains nearly unaffected. As shown in Fig. 1g, $\text{Na}_v1.9$ carries a serine residue at the critical position of the TTX-affinity site^{28,29,33}. This finding identifies $\text{Na}_v1.9$ as an obvious candidate mediating BDNF-evoked I_{Na} .

Critical roles of TrkB and $\text{Na}_v1.9$ for BDNF-evoked I_{Na}

To determine which of the two receptor tyrosine kinase candidates, TrkB or TrkC, is involved in BDNF-evoked excitation in SH-SY5Y cells, we cloned corresponding amplification products (Fig. 1e) in their antisense orientation in the eukaryotic expression vector pCAGGS³⁴. This vector allows a strong and long-term expression of RNA molecules under the control of a modified chicken β -actin promoter in combination with a cytomegalovirus (CMV) immediate-early enhancer in multiple cell types. SH-SY5Y cells were transiently transfected with either TrkB or TrkC antisense expression vectors and then, 24 h later, differentiated with 10 μM retinoic acid for an additional 72–96 h to upregulate Trk receptors

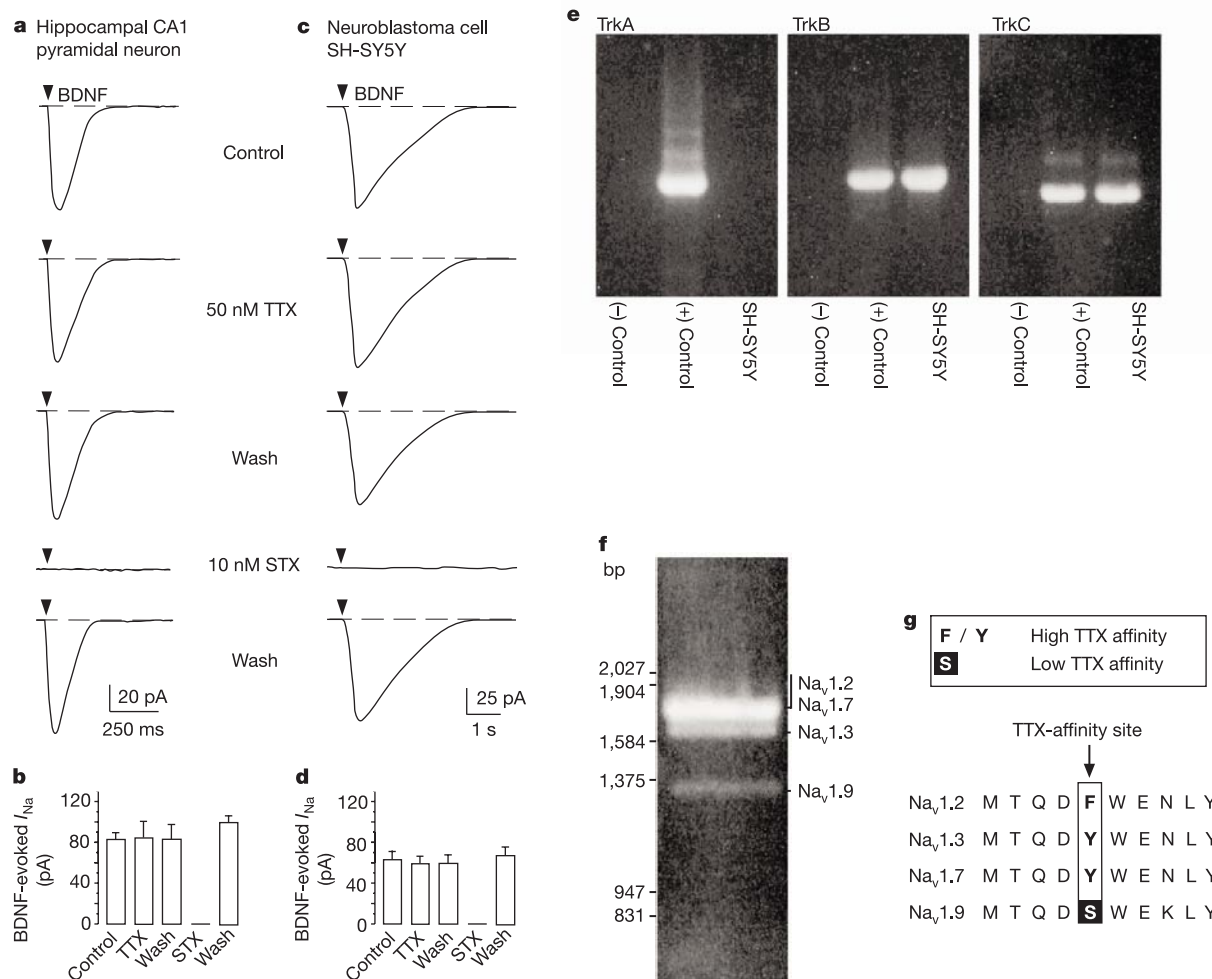


Figure 1 Pharmacological properties of the BDNF-evoked inward current (I_{Na}). **a**, Rat hippocampal CA1 pyramidal neuron. BDNF-evoked I_{Na} , evoked by applying BDNF (50 ng ml^{-1} pulse) focally for 50 ms, is insensitive to tetrodotoxin (TTX) and sensitive to saxitoxin (STX). **c**, Similar results to **a** obtained in a cultured SH-SY5Y cell. Arrowheads indicate the time of BDNF application. **b**, **d**, Plot of BDNF-evoked I_{Na} amplitudes under control conditions and after bath application of TTX and STX, in CA1 pyramidal neurons (**a**; $n = 19$, mean $\pm$ s.e.m.) and in SH-SY5Y cells (**c**; $n = 16$, mean $\pm$ s.e.m.). In all experiments the holding potential was -60 mV. **e**, RT-PCR-based identification of Trk

receptors in SH-SY5Y cells. mRNA from human brain served as a positive control ((+) control) and water served as a negative control ((-) control). **f**, RT-PCR based identification of sodium channels in SH-SY5Y cells using degenerate primers. bp, base pairs. **g**, Alignment of the TTX affinity motif in domain DI-SS2 of sodium channel members identified in SH-SY5Y cells. The serine residue (S) of $\text{Na}_v1.9$ represents the molecular motif for low TTX affinity. Tyrosine (Y) and phenylalanine (F) residues in this position of $\text{Na}_v1.2$, $\text{Na}_v1.3$ and $\text{Na}_v1.7$ mediate the high affinity for TTX.

and to reduce the cellular growth rate²⁶. As a reporter for successful transfection, enhanced green fluorescent protein (eGFP) was co-transfected in a 1:10 molar ratio. Next, patch-clamp whole-cell recordings were used to determine the effect of the antisense mRNA/eGFP expression on BDNF-evoked I_{Na} (Fig. 2a). The treatment of SH-SY5Y cells with antisense TrkB mRNA caused a nearly complete elimination ($91.36 \pm 2.75\%$ reduction, $n = 15$ cells) of BDNF-evoked I_{Na} (Fig. 2b). By contrast, eGFP expression alone (Fig. 2d) (9 out of 9 cells) or antisense TrkC (Fig. 2c) (14 out of 14 cells) had no significant effect on BDNF-evoked I_{Na} .

Subsequently we cloned the amplification product of $Na_v1.9$ and, as a control, that of the prototypical sodium channel $Na_v1.2$ (Fig. 1f) in antisense orientation in the vector pCAGGS. We performed the antisense expression in SH-SY5Y cells by using the procedures described above for TrkB and TrkC. Patch-clamp recordings from SH-SY5Y cells expressing antisense mRNA (Fig. 3a) demonstrated that antisense $Na_v1.9$ mRNA (Fig. 3b, f) entirely abolished the expression of BDNF-evoked inward current in all cells tested (22 out of 22, Fig. 3c, f). Cells expressing antisense $Na_v1.2$ mRNA (13 out of 13) or only eGFP-expressing cells (15 out of 15) responded normally to BDNF. These experiments indicate that TrkB and $Na_v1.9$ are necessary molecular constituents for BDNF-evoked I_{Na} .

Reconstitution of BDNF-evoked I_{Na} in HEK cells

To verify the above hypothesis, we aimed to reconstitute BDNF-evoked I_{Na} in non-neuronal human embryonic kidney-293 (HEK-293) cells co-expressing TrkB and $Na_v1.9$. To achieve this, we first used an RT-PCR approach to amplify the $Na_v1.9$ channel encoding

complementary DNA from SH-SY5Y cells and then cloned it under control of a CMV promoter in a pcDNA3.1 vector derivative. DNA-sequence analysis of the resulting $Na_v1.9$ primary sequence (EMBL/GenBank accession number AJ417790) revealed its identity to recently described human $Na_v1.9$ sequences (accession numbers AF109737 and AF188679 (refs 22 and 23 respectively)). To verify the integrity of the $Na_v1.9$ cDNA and the ability of the vector construct to express the $Na_v1.9$ protein, we deleted the stop codon of our expression construct and introduced an epitope tag (V5-6xHis) at the carboxy-terminal end. The $Na_v1.9$ -V5-His proteins were expressed in HEK-293 cells and analysed by immunofluorescence and western blot analysis using antibodies generated against the V5 tag. Figure 4a illustrates an example of an HEK-293 cell treated with fluorescently labelled antibodies against TrkB (top) and $Na_v1.9$ -V5-His proteins (bottom), demonstrating the ability of the corresponding cDNAs to produce TrkB and $Na_v1.9$ proteins. Similar results were obtained in all experiments ($n = 10$). The western analysis of crude urea extracts from transfected HEK-293 cells identified the $Na_v1.9$ -V5-His channel fusion protein at a roughly estimated relative molecular mass of 270,000 ($n = 3$) (Fig. 4b).

We then performed a whole-cell patch-clamp analysis of transfected HEK-293 cells. In no instance (16 out of 16 cells) did application of BDNF produce an inward current in cells that transiently expressed TrkB alone (Fig. 4c). Similarly, cells (21 out of 21) expressing the $Na_v1.9$ sodium channel alone (Fig. 4d) failed to respond to BDNF. However, in cells co-expressing TrkB and $Na_v1.9$, BDNF evoked an inward current (14 out of 14 cells) (Fig.

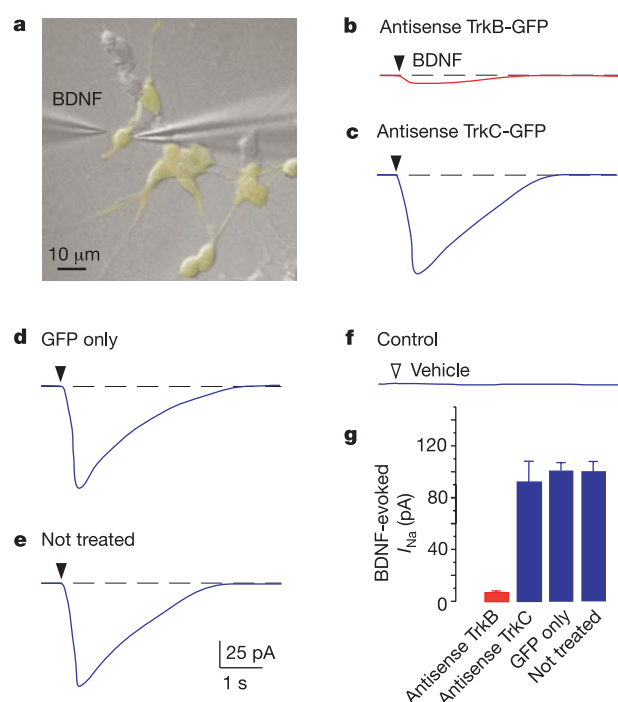


Figure 2 TrkB is required for BDNF-evoked I_{Na} in SH-SY5Y cells. **a**, Representative photomicrograph of antisense TrkB-GFP-transfected SH-SY5Y cells. The BDNF application pipette (left) and the recording patch pipette (right) are shown. **b–d**, BDNF-evoked I_{Na} recorded in SH-SY5Y cells transfected with antisense TrkB-GFP (**b**), with antisense TrkC-GFP (**c**), with GFP alone (**d**), and in untreated SH-SY5Y cells (**e**). **f**, Vehicle application failed to evoke a current. Traces are averages of three consecutive responses. In all experiments the holding potential was -60 mV and BDNF (50 ng ml^{-1} pulse) was focally applied for 50 ms. **g**, Summary plot of BDNF-evoked I_{Na} amplitudes in antisense TrkB-GFP-transfected ($n = 15$), in antisense TrkC-GFP-transfected ($n = 14$), in GFP-transfected ($n = 9$), and in untreated ($n = 32$) SH-SY5Y cells.

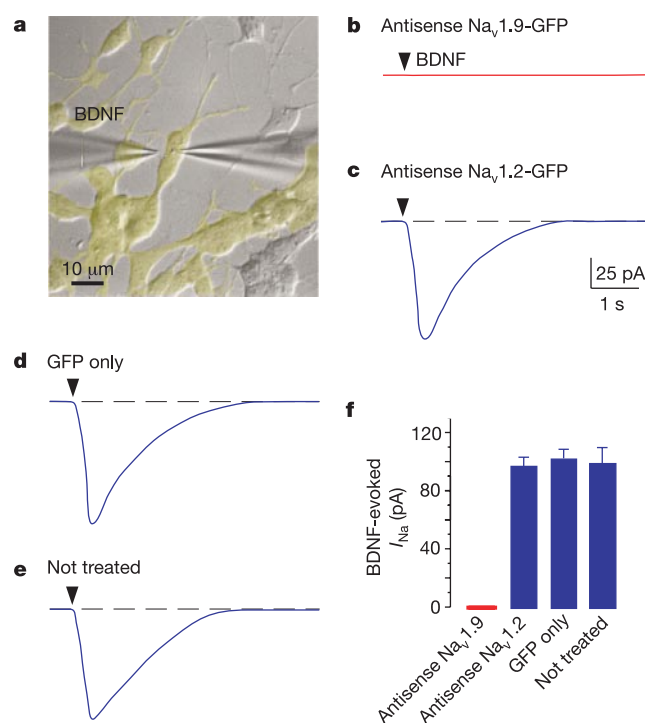


Figure 3 $Na_v1.9$ is required for BDNF-evoked I_{Na} in SH-SY5Y cells. **a**, Representative photomicrograph of antisense $Na_v1.9$ -GFP-transfected SH-SY5Y cells. **b–e**, BDNF-evoked I_{Na} recorded in SH-SY5Y cells transfected with antisense $Na_v1.9$ -GFP (**b**), antisense $Na_v1.2$ -GFP (**c**), GFP alone (**d**), and in untreated SH-SY5Y cells (**e**). Current traces are averages of three consecutive responses. In all experiments the holding potential was -60 mV and BDNF (50 ng ml^{-1} pulse) was focally applied for 50 ms. **f**, Summary plot of BDNF-evoked I_{Na} amplitudes in antisense $Na_v1.9$ -GFP-transfected ($n = 22$), $Na_v1.2$ -GFP-transfected ($n = 13$), GFP-transfected ($n = 15$), and in untreated ($n = 41$) SH-SY5Y cells.

4e). In control experiments we co-expressed the sodium channel $\text{Na}_v1.7$ (NE-Na) (accession number X82835)³² with TrkB (7 out of 7 cells). $\text{Na}_v1.7$ was selected because it is the most homologous sodium channel family member to $\text{Na}_v1.9$ in SH-SY5Y cells (see above). In HEK-293 cells co-expressing TrkB and $\text{Na}_v1.7$, local application of BDNF did not produce any response (9 out of 9 cells) (Fig. 4f). By contrast, step depolarization readily evoked the characteristic Na^+ inward current in these cells (6 out of 6 cells). Notably, HEK-293 cells expressing $\text{Na}_v1.9$, without (6 out of 6 cells) (Fig. 4d) or with TrkB (7 out of 7 cells) (Fig. 4e), failed to produce an inward current in response to depolarizing voltage steps. Depolarizing pulse protocols that were applied without success include voltage steps from -60 or -50 to -20 mV (a protocol that evoked

current in cells transfected with $\text{Na}_v1.7$), combined with hyperpolarizing pre-pulse steps to -110 mV.

Finally, we compared the reconstituted BDNF-evoked I_{Na} in HEK-293 cells with BDNF-evoked current in hippocampal CA1 pyramidal neurons. Figure 5a, b demonstrates that BDNF-evoked I_{Na} recorded in HEK-293 cells has a similar STX sensitivity and TTX insensitivity as BDNF-evoked I_{Na} found in hippocampal neurons (Fig. 1a, b). Furthermore, we analysed the voltage-dependence of BDNF-evoked I_{Na} in hippocampal (7 out of 7 cells) and in HEK-293 cells (9 out of 9 cells). In both instances (see Fig. 5c–f), BDNF-evoked I_{Na} reversed at the calculated reversal potential for Na^+ ions (E_{Na}). Moreover, in both cell types the inward current gradually decreased at membrane potentials below -65 mV and disappeared at -110 mV. This newly observed rectification of BDNF-evoked I_{Na} was not investigated further, but in the context of the main aim of this study it is important to note that the voltage-dependence together with the TTX insensitivity provide strong evidence for a

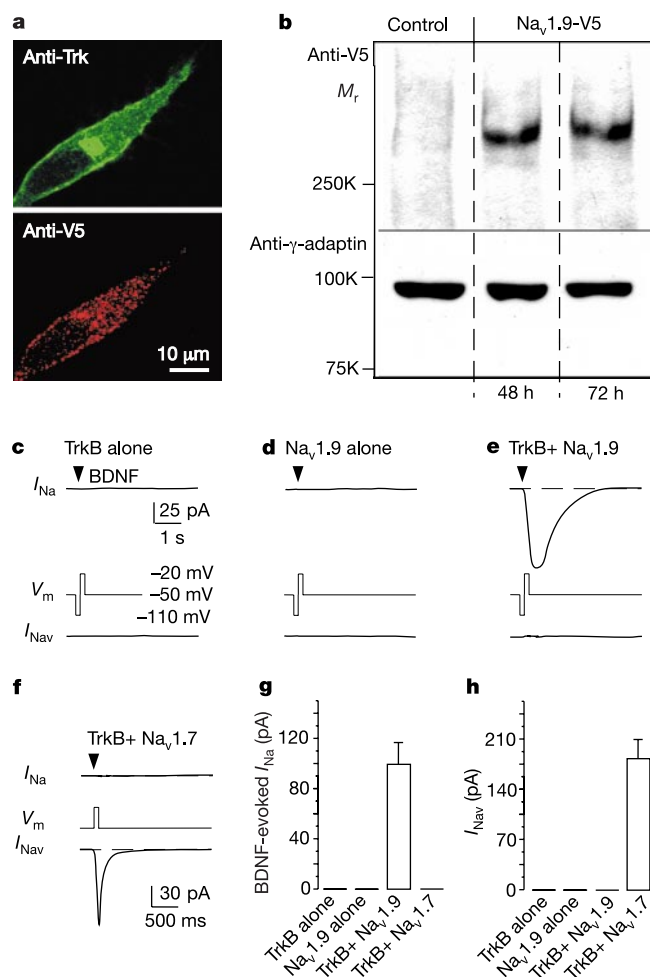


Figure 4 Reconstitution of BDNF-evoked I_{Na} in HEK-293 cells. **a**, Confocal images of immunofluorescent staining of a HEK-293 cell co-expressing recombinant TrkB receptors (top) and V5-tagged $\text{Na}_v1.9$ sodium channel protein (bottom). **b**, Western detection of $\text{Na}_v1.9$ -V5 proteins. TrkB and $\text{Na}_v1.9$ -V5 were co-expressed in HEK-293 cells for 48 or 72 h (top). Anti- γ -adaptin labels (bottom) indicate the use of equal amounts of protein ($50 \mu\text{g}$ per lane). Exposure time for γ -adaptin was 30 s, exposure time for $\text{Na}_v1.9$ -V5 was 4 h. M_r , relative molecular mass. **c–f**, BDNF-evoked current (I_{Na}) and voltage-step-induced current (I_{Nav}) in a HEK-293 cell transiently transfected with TrkB (**c**), $\text{Na}_v1.9$ (**d**), TrkB and $\text{Na}_v1.9$ (**e**), and TrkB and $\text{Na}_v1.7$ (**f**). **g, h**, Summary plots of BDNF-evoked I_{Na} amplitudes (**g**) and voltage-step-induced current amplitudes (I_{Nav}) (**h**) in HEK-293 cells transfected with TrkB (BDNF-evoked I_{Na} , $n = 16$; I_{Nav} , $n = 5$), $\text{Na}_v1.9$ (BDNF-evoked I_{Na} , $n = 21$; I_{Nav} , $n = 6$), TrkB and $\text{Na}_v1.9$ (BDNF-evoked I_{Na} , $n = 14$; I_{Nav} , $n = 7$), and TrkB and $\text{Na}_v1.7$ (BDNF-evoked I_{Na} , $n = 7$; I_{Nav} , $n = 6$). Current traces in **c–f** are averages of three consecutive responses. In all experiments the holding potential was -50 mV and BDNF (50 ng ml^{-1} pulse) was focally applied for 50 ms. Middle traces (V_m) indicate the voltage step protocol.

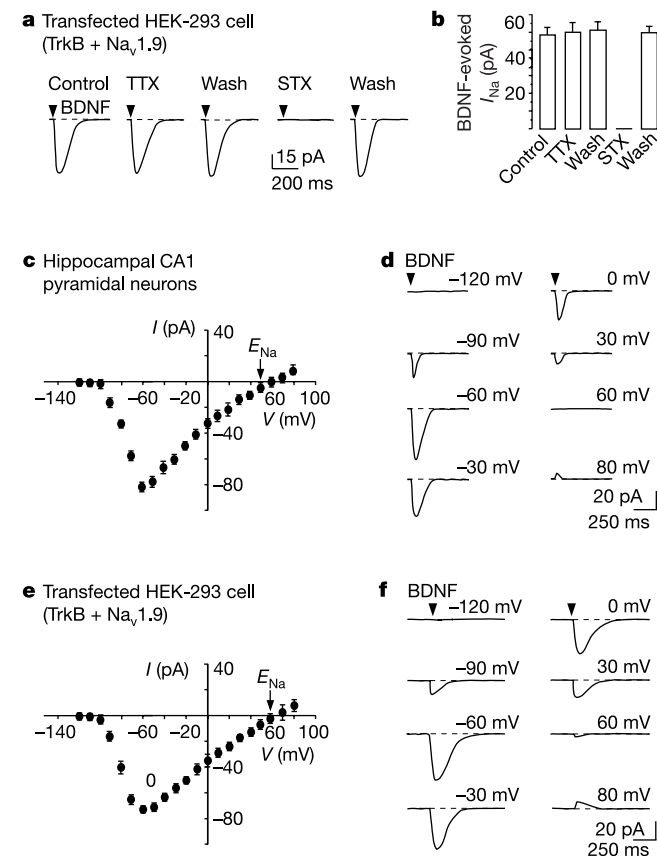


Figure 5 Comparison of reconstituted and neuronal BDNF-evoked I_{Na} . **a**, BDNF-evoked I_{Na} in an HEK-293 cell transfected with TrkB and $\text{Na}_v1.9$. BDNF-evoked I_{Na} was reversibly blocked by saxitoxin (STX; 10 nM), but not by tetrodotoxin (TTX; 50 nM). **b**, Summary plot of BDNF-evoked I_{Na} in transfected HEK-293 cells under control conditions and after bath application of TTX and STX, respectively ($n = 12$ cells for each condition). **c**, Current–voltage relationship of BDNF-evoked I_{Na} in hippocampal ($n = 7$) CA1 pyramidal cells (mean $\pm$ s.e.m.). **d**, Representative recording of BDNF-evoked I_{Na} in a hippocampal CA1 pyramidal cell at different membrane potentials, as indicated. **e**, Current–voltage relationship of BDNF-evoked I_{Na} in HEK-293 cells transfected with TrkB and $\text{Na}_v1.9$. Note that reversal potential of BDNF-evoked I_{Na} (approximately 60 mV) corresponded precisely to E_{Na} , the calculated reversal potential for sodium currents. **f**, Representative recording of BDNF-evoked I_{Na} in an HEK-293 cell transfected with TrkB and $\text{Na}_v1.9$ at different membrane potentials, as indicated. Current traces in **a, d** and **f** are averages of three consecutive responses. The holding potential was -60 mV in pyramidal neurons and -50 mV in HEK-293 cells. BDNF (50 ng ml^{-1} pulse) was focally applied for 50 ms.

functionally complete reconstitution of BDNF-evoked I_{Na} in HEK-293 cells co-expressing TrkB and $Na_v1.9$. To test whether $Na_v1.9$ is also needed in neurons for BDNF-evoked I_{Na} , we performed antisense expression in cultured hippocampal neurons by using the procedures described above for SH-SY5Y cells (see Figs 3 and 4). Patch-clamp recordings from hippocampal neurons expressing antisense mRNA demonstrated that antisense $Na_v1.9$ mRNA (Fig. 6) largely abolished the expression of BDNF-evoked inward current (Fig. 6b, d). Neurons treated only with the transfection reagent without the antisense mRNA responded normally to BDNF (Fig. 6c). Thus, these experiments verify that $Na_v1.9$ is required in neurons for BDNF-evoked I_{Na} .

Discussion

Our results identify the molecular determinants of the rapid neurotrophin-evoked neuronal depolarization. The reconstitution of the BDNF-evoked inward current in HEK-293 cells demonstrates that BDNF-evoked I_{Na} requires the combined contribution of the receptor tyrosine kinase TrkB and that of the TTX-insensitive Na^+ channel $Na_v1.9$. In view of its high sensitivity to STX, its insensitivity to TTX, and its similar voltage-dependence of activation (Fig. 5), the BDNF-evoked I_{Na} reconstituted in HEK-293 cells seems to be entirely identical to the BDNF-evoked I_{Na} recorded in neurons of the CNS. It is remarkable that the expression of just TrkB together with $Na_v1.9$ was necessary and sufficient for the reconstitution of BDNF-evoked I_{Na} in HEK-293 cells. This indicates that, if at all, ubiquitously available cellular constituents may be additional elements of the BDNF-sensing receptor-channel complex. Figure 6e illustrates our hypothetical model of this complex, in which the connecting circle stands for the (as yet unknown) site of interaction between receptor and channel. This model, which does not require a

contribution from soluble second messengers, explains the rapidity of the response as well as the finding that even during prolonged whole-cell recordings (2–3 h) there is no significant attenuation of BDNF-evoked I_{Na} (ref. 19).

BDNF-evoked neuronal excitation has been found in all types of neurons of the CNS studied so far at every developmental stage from newborn to adulthood. The types of neurons that have tested positive for this excitation include hippocampal CA1 (ref. 19), CA3 (A.K. and Y. Kovalchuk, unpublished work) and dentate granule cells¹³, cortical pyramidal cells, cerebellar granule and Purkinje cells¹⁹, as well as hippocampal interneurons (A.K. and Y. Kovalchuk, unpublished work). An emerging function of BDNF-evoked I_{Na} for synaptic plasticity was recently revealed by ref. 13. The latter study identified dendritic spines as the primary sites of BDNF-evoked I_{Na} initiation in CNS neurons. Pairing weak afferent stimuli with brief BDNF pulses, applied specifically to the active synaptic inputs, evoked LTP in hippocampal dentate granule or in CA1 pyramidal cells. This LTP occluded with the conventional, tetanic stimulation-evoked LTP, indicating that BDNF-evoked I_{Na} is not responsible for a new form of LTP, but rather represents a powerful means of rapid amplification of the induction process in an input-specific manner. Thus, the discovery of the constituents of BDNF-evoked I_{Na} provides a molecular basis for the proposed immediate and instructive role of the BDNF–TrkB system for LTP^{13,35,36}. Another role of BDNF-evoked I_{Na} was highlighted by a recent study investigating the mechanisms of neurite outgrowth. It was found that BDNF-evoked neurite outgrowth in cultured hippocampal neurons was blocked with high affinity by STX, but not by TTX³⁷. Instead, BDNF-independent neurite outgrowth was insensitive to STX. Notably, in cortical neurons, BDNF-evoked dendritic growth requires electrical activity³⁸. The requirement for both neurotrophin signalling and activity provides a mechanism for selectively enhancing the growth and connectivity of active neurons³⁸.

Previously, gating of Na^+ channels was believed to rely exclusively on changes in membrane voltage. Our results represent direct evidence that the opening of a sodium channel, $Na_v1.9$, which shares a high homology with prototypical voltage-gated sodium channels—for example, $Na_v1.2$ or $Na_v1.7$ —may be mediated by the ligand BDNF. $Na_v1.9$ is best known for its high expression levels in peripheral sensory neurons^{20,39}, a finding that led to the suggestion of its involvement in pain-related signalling^{40,41}. Further to its presence in the peripheral nervous system, $Na_v1.9$ was detected by ‘*in situ*’ and northern blot hybridization in virtually every region of the brain, including cortex, cerebellum, amygdala, thalamus and hippocampus²³. Moreover, by performing an RT-PCR examination with specific primers combined with sequence analysis, we detected full-length $Na_v1.9$ in rat cortex, cerebellum and hippocampus as well as in mouse brain (our own unpublished results). By using antisense mRNA expression we show that $Na_v1.9$ is needed in hippocampal neurons for BDNF-evoked I_{Na} . Previous suggestions concerning the cellular functions of $Na_v1.9$ in peripheral sensory neurons range from an involvement in the control of resting potential⁴² to a suggested role as a ‘window current’⁴³. These functional roles were proposed on the basis of recordings of a persistent Na^+ current in neurons, in which it was assumed that the only TTX-insensitive sodium channel was $Na_v1.9$ (ref. 44). However, the attempts to validate the functional properties of $Na_v1.9$ in HEK-293 cells remained controversial. Although it was reported that the expression of $Na_v1.9$ (old nomenclature SNS2) in HEK-293T cells produced a Na^+ current with fast kinetics³⁹, later studies^{43,44} questioned these results and reported the absence of any Na^+ current in HEK-293 cells expressing recombinant $Na_v1.9$. In our study, HEK-293 cells expressing just $Na_v1.9$ did not show any current (Fig. 4). Opening of $Na_v1.9$ channels was only achieved through application of BDNF, when $Na_v1.9$ channels were expressed together with the receptor tyrosine kinase TrkB. Gating

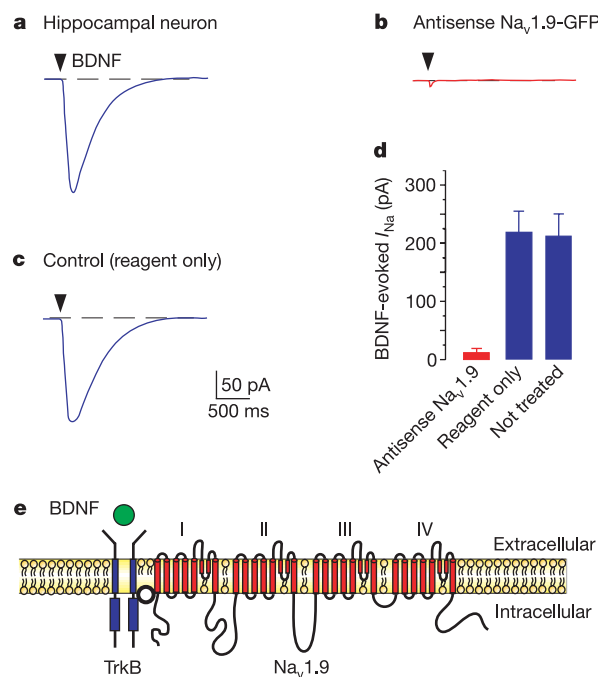


Figure 6 $Na_v1.9$ is required for BDNF-evoked I_{Na} in hippocampal neurons. **a–c**, BDNF-evoked I_{Na} recorded in untreated hippocampal neurons (**a**), in neurons transfected with antisense $Na_v1.9$ -GFP (**b**), and in neurons treated with just the transfection reagent (**c**). **d**, Summary plot of BDNF-evoked I_{Na} amplitudes (pA $\pm$ s.e.m.) in antisense $Na_v1.9$ -GFP-transfected neurons ($n = 9$), in neurons treated with the transfection reagent ($n = 4$), and in untreated ($n = 7$) neurons. Current traces are averages of three consecutive responses. In all experiments the holding potential was -60 mV and BDNF (50 ng ml $^{-1}$ pulse) was focally applied for 50 ms. **e**, Hypothetical model of the TrkB– $Na_v1.9$ complex.

by voltage steps, which was completely absent under our conditions, may require the expression of additional subunits and/or regulatory factors, such as annexin II light chain⁴⁵. The general profile of the current–voltage relation of BDNF-evoked I_{Na} , with a reduced or absent BDNF-mediated channel opening at very negative membrane potentials (Fig. 5c, e), may suggest that receptor-mediated activation requires ‘voltage-priming’ of the $Na_v1.9$ channel. Our results demonstrate that $Na_v1.9$ is a unique type of sodium channel that is gated, at least in CNS neurons, by extracellular ligands rather than by voltage. These ligands are the neurotrophins BDNF or neurotrophin-4/5, the most powerful endogenous neuroexcitants reported so far¹⁹. □

Methods

Cell culture

Human SH-SY5Y neuroblastoma and HEK-293 cells (German Collection of Microorganisms and Cell Cultures) were cultured at 37 °C with 5% CO₂ in DMEM supplemented with 10% heat-inactivated fetal bovine serum. For electrophysiological recordings, cells were grown on plastic coverslips (Sarstedt).

RT–PCR screening

mRNA was reverse transcribed using random hexamers and Superscript II (Life technologies). For the multiple display analysis of sodium channels, degenerate primers were deduced from highly conserved sequence motifs of the voltage-gated sodium channel family members $Na_v1.1$ to $Na_v1.9$. The upper primer (5′-ATGACNCARGAYTCTGGGA-3′) represented the primary sequence motif MTQD(Y/F/C/S)WE of the DI-SS2 domain. The lower primer (5′-CCCACATRKTYTCGATCCA-3′) was deduced from the sequence motif WIE(N/T)MW in domain DII-SS2. Amplification of cDNA fragments was performed with the Expand High Fidelity PCR system (Roche). The Trk receptor expression profile of SH-SY5Y cells was determined by standard RT–PCR procedures with specific primers. Amplification products were cloned into pT-Adv (Clontech) and identified by DNA sequencing.

mRNA antisense expression

cDNA fragments representing the receptor domain of human TrkB and TrkC were amplified from SH-SY5Y mRNA using the primers: TrkB forward, 5′-TCGAATTCGCCCAATGTCGTCCTGGAT AAGGTG-3′; TrkB reverse, 5′-TCGAATTCGGTGTCCCCGATGTCATTC-3′; TrkC forward, 5′-TCGAATTCCTTGGCCAGCCAAAGTGTAG-3′; TrkC reverse, 5′-TCGAATTCGCCACGGGACCCCTTCATTC-3′ (engineered *EcoRI* sequences are underlined). Amplification products were cloned in the antisense orientation in the *EcoRI* site of the expression vector pCAGGS that carries a CMV-immediate early enhancer/chicken β -actin promoter. cDNA fragments of the sodium channel α -subunits of human $Na_v1.2$ and $Na_v1.9$ were rescued from their corresponding pT-Adv vectors (see above) and also cloned into the *EcoRI* site of pCAGGS. Resulting antisense constructs and a pCAGGS-eGFP vector (transfection control) were co-transfected in SH-SY5Y cells within a molar ratio of 10:1 using Effectene reagent (Qiagen). Twenty-four hours after transfection, 10 μ M all-*trans* retinoic acid was added for 72–96 h to upregulate TrkB receptors. For antisense mRNA expression in neurons, a PCR fragment of rat $Na_v1.9$ (EMBL/GenBank accession number AF059030, base pairs 1103–2338) was cloned in the antisense direction into the *EcoRI* site of the vector pcDNA3 (Invitrogen). Dissociated rat hippocampal neurons were cultured for 4 days in neurobasal medium (Invitrogen). Then, these cells were co-transfected with the pcDNA3 rat $Na_v1.9$ antisense construct and a pCAGGS-eGFP vector in a molar ratio of 20:1 using Lipofectamine 2000 (Invitrogen). Antisense mRNA and eGFP were expressed for 72 h and then analysed by using whole-cell recordings.

Cloning and expression of $Na_v1.9$ and TrkB

Full-length human $Na_v1.9$ (EMBL/GenBank accession number AF109737) was cloned from three partial cDNA PCR fragments. $Na_v1.9$ fragment 1 (nucleotides 200–1152) was cloned into pcDNA3.1-V5-His-TOPO (Invitrogen). Fragments 2 (nucleotides 1072–3660) and 3 (nucleotides 3559–5618) of $Na_v1.9$ were subcloned into pT-Adv (Clontech), sequenced and then fused with fragment 1 using the restriction sites *EcoRI* (position 1124), *BspEI* (position 3636) and *XbaI* (artificially introduced at the 3′ end). Stable propagation of full-length $Na_v1.9$ expression vectors in *Escherichia coli* XL10 (Stratagene) was possible after exchange of the *ColEI* origin in pcDNA3.1-V5-His-TOPO against the *ColEI*-origin/*rop* gene derivative of pBR322. The resulting construct was sequenced by MWG-biotech (publication quality) and the sequence submitted to the EMBL database (accession number AJ417790). For detection of $Na_v1.9$ proteins, a 3′ terminal PCR fragment of $Na_v1.9$ was fused in frame with a V5-6 $\times$ His epitope. A cDNA corresponding to full-length TrkB was amplified from mRNA using a standard PCR approach and cloned into the *EcoRI* site of pCAGGS. For electrophysiological recordings, HEK-293 cells were transiently transfected using Effectene (Qiagen).

Slice preparation and electrophysiology

Hippocampal slices (250 μ m) were prepared from 9–14-day-old Wistar rats as previously reported. In brief, after anaesthesia the animals were decapitated, the tissue was rapidly removed and placed in ice cold artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, 20 glucose (all from

Sigma) bubbled with 95% O₂/5% CO₂. After sectioning, slices were kept in ACSF for 30 min at 37 °C and then at 25 °C for up to 7 h. Whole-cell recordings were performed from cell bodies located near the top surface of slice preparations or from cultured cells with an EPC9 amplifier (HEKA) using ‘Pulse’ software (HEKA) for data acquisition. Pipettes were pulled from borosilicate glass (Hilgenberg) and coated with silicone (RTV 615, GE Silicons). The intracellular solution contained (in mM): 135 CsCl, 15 NaCl, 0.6 EGTA, 5 tetraethylammonium ion, 2 MgCl₂, 10 HEPES, 0.4 GTP, 4 Mg-ATP, pH 7.3. During the experiments, the slices and the cultured cells were continuously perfused with ACSF (20–22 °C). Except for obtaining data for the *I*–*V* curves, holding potentials were generally set to –60 mV. BDNF (Sigma) was diluted in ACSF and was usually applied by a picospritzer (Parker) coupled to standard micropipettes. The application pipettes were placed at a distance of 5–15 μ m above the soma. All plastic and glassware was blocked twice with PBS containing 0.1% BSA (Sigma) before exposing the material to BDNF to prevent binding of BDNF to the storage and application ware.

SDS–PAGE and immunoassays

$Na_v1.9$ was transiently expressed for 24–72 h in HEK-293 cells. Then, cells were lysed in urea lysis buffer (8 M urea, 100 mM NaH₂PO₄, 10 mM Tris, pH 8.0, Protease Inhibitor Set) for 30 min at 0 °C. Crude protein extracts (50 μ g per lane) were analysed by SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotted on nitrocellulose (Schleicher and Schuell). For detection, proteins were probed with monoclonal antibodies against the V5 epitope (Invitrogen) and detected via horseradish-peroxidase-conjugated anti-mouse immunoglobulin- γ (IgG) using the ECL-Plus detection system (Amersham-Pharmacia). As a loading control, an antibody against γ -adaplin (Sigma) was used. For immunofluorescence, after 24 h, HEK-293 cells were fixed (4% paraformaldehyde/PBS, 15 min) and permeabilized (0.1% Triton X-100, PBS, 10 min). Cells were blocked with blocking buffer (10% goat serum, 1% milk powder, 0.1% Triton X-100, 1 h), probed with mouse anti-V5 and finally labelled with Cy5 goat anti-mouse IgG (Jackson Laboratories) in blocking buffer. Co-expressed TrkB were detected with rabbit anti-Trk (C14, Santa Cruz) and Alexa-488 goat anti-rabbit IgG (Molecular Probes) as secondary antibody. Cells were mounted in Prolong mounting medium (Molecular Probes) and imaged by confocal microscopy.

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A star in a 15.2-year orbit around the supermassive black hole at the centre of the Milky Way

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Many galaxies are thought to have supermassive black holes at their centres¹—more than a million times the mass of the Sun. Measurements of stellar velocities^{2–7} and the discovery of variable X-ray emission⁸ have provided strong evidence in favour of such a black hole at the centre of the Milky Way, but have hitherto been unable to rule out conclusively the presence of alternative concentrations of mass. Here we report ten years of high-resolution astrometric imaging that allows us to trace two-thirds of the orbit of the star currently closest to the compact radio source (and massive black-hole candidate) Sagittarius A*. The observations, which include both pericentre and apocentre passages, show that the star is on a bound, highly elliptical keplerian orbit around Sgr A*, with an orbital period of 15.2 years and a pericentre distance of only 17 light hours. The orbit with the best fit to the observations requires a central point mass of $(3.7 \pm 1.5) \times 10^6$ solar masses ($M_\odot$). The data no longer allow for a central mass composed of a dense cluster of dark stellar objects or a ball of massive, degenerate fermions.

For the past ten years we have been carrying out high-resolution near-infrared imaging and spectroscopy of the central few light years of our Milky Way for a detailed study of the stellar dynamics in the vicinity of the compact radio source Sgr A* (refs 2, 3, 5, 7), the most likely counterpart of the putative black hole^{9,10}. From a statistical analysis of the stellar proper motions (velocities on the plane of the sky derived from multi-epoch imaging data) and line-of-sight velocities (Doppler motions derived from spectral lines) we deduced the presence of a mass of about 2.6 to 3.3 million $M_\odot$ concentrated within ten light days of Sgr A* (refs 2, 3, 5). To further improve the sensitivity (by about 20) and the angular resolution/astrometric precision of our study (by about 3), we began this year to use the new COudé near-infrared camera (CONICA)/Nasmyth adaptive optics system (NAOS) imager/spectrometer on the 8-m UT4 (Yepun) of the European Southern Observatory (ESO) Very

Large Telescope (VLT)^{11–13}. Figure 1 shows a diffraction-limited (56 milli-arcseconds (mas) full-width at half-maximum, FWHM) K_s-band (2.18 μ m) image of the central 40'' of the Milky Way taken with NAOS/CONICA in May 2002. A key factor in constraining the mass distribution is the alignment of the infrared images, where the stars are observed, with the astrometrically accurate radio images, where the

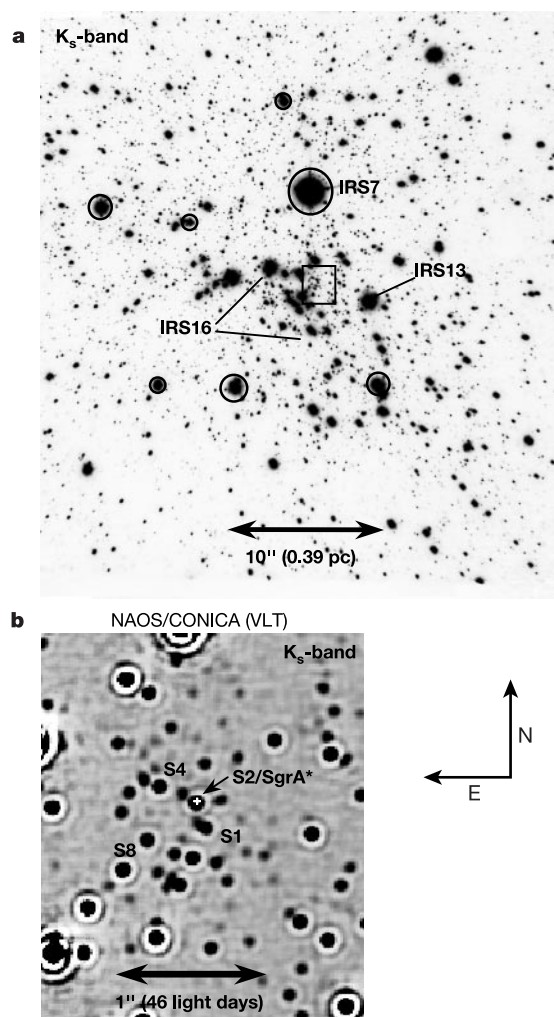


Figure 1 K_s-band image of the centre of the Milky Way. **a**, Diffraction-limited (56-mas FWHM) K_s-band (2.18 μ m) image of the central 40'' of the Milky Way, obtained with the NAOS/CONICA adaptive optics imager on UT4 (Yepun) of the VLT on 3 May 2002. The prominent infrared sources IRS7, IRS13 and IRS16 are indicated. The scale bars are for an assumed distance of 8 kpc (26,000 light years)^{5,21}. The unique infrared wavefront sensor was used to close the loop of the adaptive optics system on the bright supergiant IRS 7, about 6'' north of Sgr A*. The Strehl ratio is over 40%. The radio positions of seven SiO maser stars (open circles) were used to align the infrared image with the radio astrometry frame²³. The SiO masers originate in the central ~ 1 mas of the circumstellar envelopes of infrared bright, red giants/supergiants. The radio-to-infrared registration is accurate to ± 10 mas (including the effect of variation of the point spread function across the field), a factor-of-three improvement over ref. 14. There we could only use two SiO sources, giving the centre position, rotation angle and a single pixel scale for the infrared images. Our new analysis allows solving, in addition, for second-order imaging terms (small for NAOS/CONICA, but significant for the earlier SHARP/NTT data). **b**, The central $\sim 2''$ region (rectangle in **a**) around the compact radio source Sgr A* (cross). This image is a sum of images taken in May 2002 and has been deconvolved with a linear Wiener filter method to remove the seeing halos. The ring structures around the brighter stars are artefacts of the linear deconvolution algorithm that arise because information on the point spread function in Fourier space is not known up to infinite frequencies. Several of the stars near Sgr A* are marked, including the closest star at present (S2) (ref. 5).

where SgrA* is observed. For this purpose we aligned our NAOS/CONICA images with the astrometric grid using seven SiO maser sources in the field of view (circles in Fig. 1) whose positions are known through measurements with the Very Large Array (VLA) and the Very Long Baseline Array (VLBA) to accuracies of a few mas²³. Having thus derived astrometric infrared positions for 2002, we were then able to compute exact stellar positions relative to SgrA* (in right ascension and declination) for all epochs (including data taken with the SHARP camera at the ESO New Technology Telescope, NTT) between 1992 and 2002. The resulting position of the radio source SgrA* on the infrared image has a 1σ uncertainty of ± 10 mas, or about a factor of three better than previously¹⁴. The new position of SgrA* is around 50 mas east of the position given in ref. 14. In spring 2002 the orbiting star S2 had approached SgrA* to within 10–20 mas, thus providing a unique opportunity to determine the mass a factor of 10–20 times more closely in than in previous work.

The first measurements of orbital accelerations for S2 and S1, the two stars closest to SgrA*, were consistent with orbits bound to a central object of about 3 million $M_{\odot}$, but still allowed a wide range of possible orbital parameters^{6,7}. Specifically, possible orbital periods for S2 ranged from 15 to 500 yr (ref. 6). With our new data, we are now able to determine a unique orbit for S2 from astrometric proper motions and provide strong constraints on the mass distribution on distances less than one light day. Figure 2 shows the measured 1992–2002 positions of S2 relative to SgrA*. In spring 2002 we happened to catch the pericentre passage of the star, at which point the measured velocity exceeded $5,000 \text{ km s}^{-1}$, about

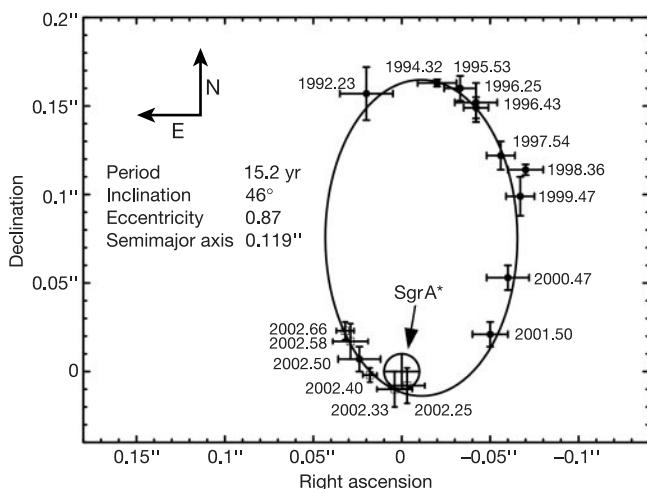


Figure 2 Orbit of S2 around SgrA*. Orbit of S2, relative to the position of SgrA* (large cross and circle, denoting the ± 10 mas uncertainties of the infrared-radio astrometry). The filled small circles (with 1σ errors) between 1992 and 2001, and at 2002.50, denote the results of our speckle imaging with the SHARP camera on the ESO NTT^{5,7}. The five open rectangles are the NAOS/CONICA data points on 2002.25, 2002.33, 2002.40, 2002.58 and 2002.66. The projection of the best-fitting Kepler orbit is shown as a continuous curve, with its main parameters listed adjacent to the orbit (see also Table 1). For determination of the orbital elements (Table 1) we used the publicly available Binary Star Combined Solution Package¹⁵, which computes best-fit orbits from position–time series (including their errors). Uncertainties in the derived parameters are determined from a covariance matrix analysis. The additional uncertainty introduced by the astrometric errors is of similar size and was estimated by letting the position of SgrA* vary randomly within its 1σ astrometric uncertainty limits and examining the change in the resulting orbital parameters. Because we do not know the line-of-sight velocity of the star, the sign of the inclination given in Table 1 is undetermined. The angle of the line of nodes (36°) is counted east of north. The angle from node to pericentre is counted from the node in the northeast quadrant in the direction of the motion of S2.

eight times greater than $7.6 \text{ yr ago}^{2,3}$ when S2 was at apocentre. The S2 data points trace two-thirds of a closed orbit and are robustly fit by a bound keplerian orbit around a central point mass located at the position of SgrA*. The parameters of the best-fitting orbit, along with their fit and astrometric errors, are given in Table 1. They were derived using the publicly available Binary Star Combined Solution Package¹⁵. For the nominal SgrA* position, the uncertainties of the fit parameters are generally less than 10%. The additional uncertainty introduced by the astrometric errors is of similar size. The semimajor axis ($a = 5.5$ light days) and orbital period (15.2 yr) imply a mass of $(3.7 \pm 1.5) \times 10^6 M_{\odot}$ within the pericentre radius of 124 AU, or 17 light hours. The pericentre passage of S2 in April/May 2002 thus probes the mass concentration at around 2,100 times the Schwarzschild radius of a $3 \times 10^6 M_{\odot}$ black hole. The pericentre distance radius of S2 is 70 times greater than the distance from the black hole, where the star would be disrupted by tidal forces (about 16 light minutes for a $\sim 15 M_{\odot}$, $7 R_{\odot}$ star like S2; ref. 3). Because tidal energy deposition falls faster than the sixth power of the ratio of tidal radius to orbital radius, tidal effects near the perinigricon of S2 are expected to be negligible, consistent with its lack of infrared variability.

The remarkable consequence of the orbital technique is that the mass can be determined from a single stellar orbit, in comparison to the statistical techniques that use several tens to hundreds of stellar velocities at 10 to 300 light days from SgrA* (Fig. 3). In addition, the orbital technique requires fewer assumptions than the other estimates (for example, equilibrium and isotropy of orbits), and thus is less vulnerable to systematic effects.

The Galactic Centre mass distribution resulting from all available data is well fitted by the combination of a $(2.6 \pm 0.2) \times 10^6 M_{\odot}$ point mass (the supermassive black hole), plus the visible stellar cluster of core radius 0.34 pc, an outer power-law density distribution with exponent $\alpha = 1.8$ and central density $3.9 \times 10^6 M_{\odot} \text{ pc}^{-3}$ (Fig. 3). If the central point mass is replaced by a Plummer mass distribution, which is the most compact one expected realistically (with a power-law index of $\alpha = 5$, in order to mimic the flatness of the observed mass distribution over three orders of magnitude in radius⁵), its central density would have to exceed $10^{17} M_{\odot} \text{ pc}^{-3}$, more than four orders of magnitude greater than previous estimates^{5–7}. Such a Plummer distribution would be appropriate if the dark mass consisted of a dark cluster of low-mass stars, neutron stars or stellar black holes. The maximum lifetime of such a cluster mass against collapse (to a black hole) or evaporation would be less than a few 10^5 yr (ref. 16), clearly a highly implausible configuration. Further, theoretical simulations of very dense, core-collapsed clusters predict much shallower, near-isothermal density distributions ($\alpha \approx 2$, see discussion in ref. 3). We conclude that such a dark cluster model can now be safely rejected. Our new data also robustly exclude one of two remaining ‘dark particle matter’ models as alternatives to a supermassive black hole, namely a ball of heavy ($10\text{--}17 \text{ keV } c^{-2}$) fermions (sterile neutrinos, gravitinos or axinos) held up by degeneracy pressure^{17,18}, which in principle could account for the entire range of dark mass concentrations in galactic

Table 1 Derived orbital parameters for S2

Parameter	Value	Formal error*	Astrometric error†
Black hole mass ($10^6 \times M_{\odot}$)	3.7	1.0	1.1
Period (years)	15.2	0.6	0.8
Time of pericentre passage (years)	2002.30	0.01	0.05
Eccentricity	0.87	0.01	0.03
Angle of line of nodes (degrees)	36	5	8
Inclination (degrees)	± 46	3	3
Angle of node to pericentre (degrees)	250	4	3
Semi-major axis (mpc)	4.62	0.39	0.43
Separation of pericentre (mpc)	0.60	0.07	0.15

*The 1σ errors result from the orbital fit.

†The errors due to the 10-mas astrometric uncertainty. See Fig. 2 legend for a description of the angles and of the errors.

nuclei with a single physical model. Because of the finite size ($\sim 0.9''$ diameter) of a non-relativistic, $3 \times 10^6 M_\odot$ ball of around 16 keV fermions, the maximum (escape) velocity is about $1,700 \text{ km s}^{-1}$ and the shortest possible orbital period for S2 in such a fermion ball model would be about 37 yr (ref. 18), clearly inconsistent with the orbit of S2. The enclosed mass at perinigricon would require a neutrino mass of over 50 keV, a value which can safely be excluded for neutrino ball models trying to explain the entire range of observed masses in galactic nuclei^{17,18}. The only 'dark particle matter' explanation that cannot be ruled out by the present data is a ball of bosons, because such a configuration would have a radius only a few times greater than the Schwarzschild radius of a black hole^{16,19}. However, it would be very hard to understand how the bosons first manage to reach such a high concentration, and then avoid forming a black hole by baryonic accretion^{16,19}. The data on the Galactic Centre thus show that the central mass distribution is remarkably well described by the potential of a point mass over three orders in magnitude in spatial scale, from 0.8 light days to 2 light years. The contribution of the extended stellar cluster around SgrA* to the total mass cannot be more than a few hundred solar masses within the pericentre distance of the orbit of S2.

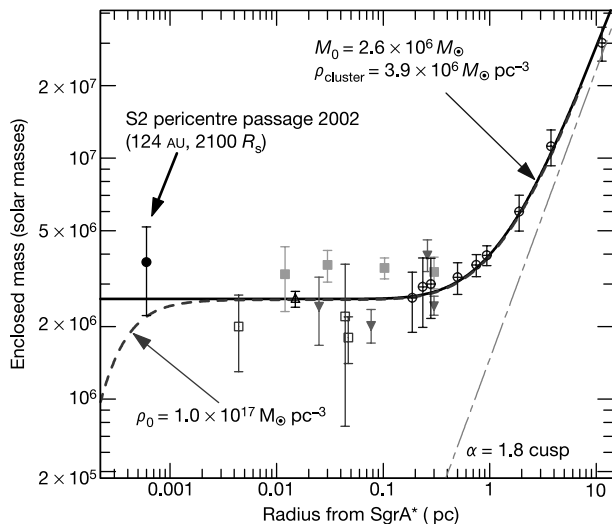


Figure 3 Mass distribution in the Galactic Centre. Mass distribution in the Galactic Centre (for an 8 kpc distance²¹). The filled circle denotes the mass derived from the orbit of S2. The error bar combines the orbital fit and astrometry errors (Table 1). Filled downward-pointing triangles denote Leonard–Merritt projected mass estimators from a new NTT proper motion data set (T.O., R.S., R.G. and A.E., manuscript in preparation), separating late and early type stars, and correcting for the volume bias in those mass estimators by scaling with correction factors (0.88–0.95) determined from Monte Carlo modelling of theoretical clusters⁵. An upward-pointing rectangle denotes the Bahcall–Tremaine mass estimate obtained from Keck proper motions⁴. Grey filled rectangles are mass estimates from a parameterized Jeans-equation model from ref. 5, including anisotropy and differentiating between late and early type stars. Open circles are mass estimates from a parameterized Jeans-equation model of the radial velocities of late type stars, assuming isotropy⁵. Open rectangles denote mass estimates from a non-parametric, maximum likelihood model, assuming isotropy and combining late and early type stars²². The different statistical estimates (in part using the same or similar data) agree within their uncertainties but the variations show the sensitivity to the input assumptions. In contrast, the new orbital technique for S2 is much simpler and less affected by the assumptions. The continuous curve is the overall best-fit model to all data. It is a sum of a $(2.6 \pm 0.2) \times 10^6 M_\odot$ point mass, plus a stellar cluster of central density $3.9 \times 10^6 M_\odot \text{ pc}^{-3}$, core radius 0.34 pc and power-law index $\alpha = 1.8$. The 'long dash, short dash' curve shows the same stellar cluster separately, but for an infinitely small core (that is, a 'cusp'). The thick dashed curve is the sum of the visible cluster, plus a Plummer model of a hypothetical concentrated ($\alpha = 5$), very compact ($R_0 = 0.00019 \text{ pc}$) dark cluster of central density $1 \times 10^{17} M_\odot \text{ pc}^{-3}$.

Thus we have presented the first step in a new phase of near-infrared observations of the immediate surroundings of the central dark mass in the centre of the Milky Way. The observation of orbits of stars surrounding the central dark object offers a clean new way of constraining its mass distribution and testing the supermassive black hole model with the simple assumption of keplerian orbits. Within the next years we hope to observe the accelerations and orbits of several faint stars near SgrA* that have become observable with the increased resolution and sensitivity of the NAOS/CONICA camera/adaptive optics system at the VLT. Even more detailed observations of the SgrA* environment will become possible with infrared interferometry at the Large Binocular Telescope, the ESO Very Large Telescope Interferometer and the Keck interferometer, which will provide resolution of a few to 10 mas (a few light hours). These offer exciting prospects for the exploration of relativistic motions at 10–100 Schwarzschild radii from the central black hole²⁰. □

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Simulation of the atmospheric thermal circulation of a martian volcano using a mesoscale numerical model

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Mesoscale (<100 km) atmospheric phenomena are ubiquitous on Mars, as revealed by Mars Orbiter Camera images^{1–3}. Numerical models provide an important means of investigating martian atmospheric dynamics, for which data availability is limited. But the resolution of general circulation models, which are traditionally used for such research, is not sufficient to resolve mesoscale phenomena^{4–6}. To provide better understanding of these relatively small-scale phenomena, mesoscale models have recently been introduced^{7–9}. Here we simulate the mesoscale spiral dust cloud observed over the caldera of the volcano Arsia Mons by using the Mars Regional Atmospheric Modelling System. Our simulation uses a hierarchy of nested models with grid sizes ranging from 240 km to 3 km, and reveals that the dust cloud is an indicator of a greater but optically thin thermal circulation that reaches heights of up to 30 km, and transports dust horizontally over thousands of kilometres.

The Mars Regional Atmospheric Modelling System (MRAMS) is derived from a widely used atmospheric simulation code for the Earth's atmosphere¹⁰. The core dynamics of the model are built around discretized primitive equations of the atmosphere that include non-hydrostatic processes and acoustic waves. The vertical coordinate (σ -z) closely follows the terrain elevation at low levels, and gradually distorts with height to a horizontally flat surface. Two-way interactive nested computational grids allow higher resolution of physical processes over limited areas. The solution obtained on a nested grid is averaged back to the coarser parent grid so that larger-scale dynamics are influenced by smaller-scale processes.

Three improvements to the MRAMS code have been made for this study. First, a new algorithm was used to calculate the horizontal pressure gradient. This method greatly reduces the numerical instability that can occur in terrain-influenced σ -z models with extremely steep topography. The addition of this code was a requirement for this study, given the topographic relief of the volcanic Tharsis region. Second, we added parameterizations to model the effect of topographic shadowing and slope angle on both the surface energy budget and the solar heating of the atmospheric column. This addition is also crucial for this simulation, because the solar heating varies significantly owing to large variations in Sun and slope geometry. The final improvement was the inclusion of a dust lifting parameterization¹¹—the mass of lifted dust increases as surface wind stress increases, and is activated when surface wind stress exceeds 14 mN.

The simulation period was three martian days, starting at a solar longitude of 180° (the vernal equinox), which corresponds to the date the cloud was imaged (Fig. 1) by the Mars Orbiter Camera (MOC). Only the last martian day of data was used for this analysis in order to minimize issues related to model spin-up. There are four, two-way interactive nested grids roughly centred over Arsia Mons. The two outer grids cover most of the Tharsis region and resolve the volcanic peaks reasonably well (Fig. 2). Note that the height of Olympus Mons and Arsia Mons are respectively 19,550 m and 16,410 m on grid two of the model, whereas observations indicate

heights of 21.3 km and 17.6 km, respectively. The topography on grid one is better resolved by at least a factor of two compared to a typical general circulation model (GCM). The domain of the third grid is slightly larger than Arsia Mons (not shown), and the fourth grid covers the caldera (Fig. 3). The horizontal grid spacing is respectively 240 km, 60 km, 15 km and 3 km for each of the nested grids. The nominal vertical grid spacing is 50 m in the lowest model level, and is stretched gradually to a maximum spacing of 1,200 m. The model top is at 60 km. The initial large-scale fields and time-dependent boundary conditions were supplied by simulation 9974 in the NASA Ames Mars General Circulation Model (MGCM) Climate Catalog. The model domain is nearly hemispheric in order to minimize the spurious influence of inaccurate boundary conditions⁸. Data from the last year of the two-year MGCM simulation was used for this purpose. The background dust concentration is fixed at an optical depth of 0.3 at 611 Pa. The lifted dust augments the background value, and total dust optical depth is used to calculate the radiative heating. The lifted dust is assumed to have a backscatter coefficient of 2.0, which is consistent with a particle diameter size of approximately 1 μ m.

The MRAMS-simulated horizontal wind field at 24 m above ground level, and the vertically integrated dust mass, is shown in Fig. 3, which can be compared to the observed cloud (Fig. 1). Although the simulated dust cloud is off-centre from the caldera, there are notable similarities between the observed and simulated cloud, and we are confident that the model has captured the essential features of the observed cloud and the accompanying atmospheric circulation. The scale of the observed and simulated clouds are approximately the same, and the sense of rotation implied by the spiral formation in the observations agrees with the clockwise circulation in the simulation. The cloud was observed at approximately 14:00 local time, and was at an altitude between 10 km and 15 km above the caldera on the basis of viewing geometry and shadow length. The uncertainty results from the pixel resolution in the image. It is not known at what time the cloud became optically visible. The simulated cloud is well organized by 16:00 local time. The cloud top is approximately 15 km above ground, in agreement with the altitude computed by the shadow geometry. The

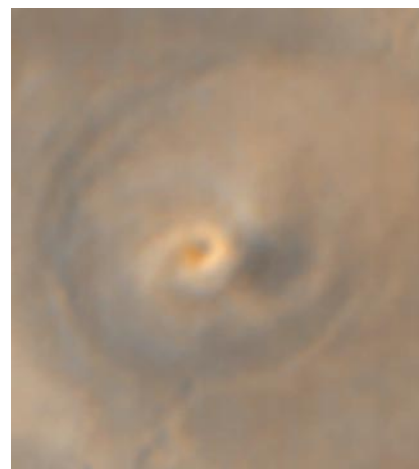


Figure 1 Mars Orbiter Camera image of a spiral dust cloud observed above the caldera of Arsia Mons. The pseudo-colour image was obtained by combining the red- and blue-filter MOC data. The original image was cropped to focus on the caldera, which is the faint, dark, ring towards the edge of the image. The cloud structure (the central, bright spiral) implies a clockwise and convergent circulation, and casts (to its right) a shadow on the caldera floor. Faint, white water ice clouds are also visible. The image should be compared with Fig. 3. Image reproduced with permission from Malin Space Science Systems.

simulated cloud is sufficiently dense to be visible through the red filter of the MOC. The spiral cloud did not form when an identically configured simulation was performed at a solar longitude of 142° . Consequently, the simulation indicates at least some seasonality to the circulation, which probably results either from changes in the thermal circulation due to changes in solar heating, or as a result in changes of the large-scale flow as predicted by the MGCM.

Although the dust cloud is visually captivating, the unseen

thermal circulation predicted by MRAMS—and from which the spiral cloud originates—is of greater interest. A vertical cross-section (Fig. 4) reveals that the spiral dust cloud is the dense portion of a much larger, optically thin, mushroom-shaped dust cloud associated with the thermal circulation forced by Arsia Mons. Parcels of air originating near the base or along the slopes of Arsia Mons traverse a helical convergent path upwards, with a peak vertical ascent rate of several metres per second. There is sufficient energy for an air parcel rising along the slopes of the volcano to ascend to an altitude of 30 km above the reference geoid. Dust is preferentially lifted near the base of the mountain, where densities are higher and dust lifting mechanisms are more effective for a given wind speed. The origin of the dust in the observed cloud is not from within the caldera, but from along the lower slopes of the volcano. The convergent wind field concentrates the dust into the optically thick spiral cloud feature.

The flow becomes horizontally divergent near the top of the circulation, where the outflow branches of the circulation transport dust laterally up to a thousand or more kilometres on either side of the volcano. Owing to the lower density at higher altitudes, conservation of mass requires that the velocity of the diverging air greatly exceed the velocity of the low-level inflow. Wind speeds along the slopes of the volcano are in excess of 25 m s^{-1} , but 15 m s^{-1} is more typical. The outflow speed aloft exceeds 40 m s^{-1} . Dust undergoing horizontal transport in the outflow branches of the thermal circulation would, in reality, undergo size sorting by way of sedimentation. A dust particle of less than $1 \mu\text{m}$ in diameter falling at 1 cm s^{-1} would require in excess of 20 martian days to fall 20 km in altitude in the absence of vertical motion in the atmosphere. Such a particle could travel significant horizontal distances within this timeframe. Dust less than $1 \mu\text{m}$ in diameter would remain aloft for even longer periods, and could easily circle the planet given only the moderate wind speeds predicted by Mars GCMs.

The air in the rising branch of the thermal circulation is cooled adiabatically to temperatures as low as 135 K at an altitude of 30 km

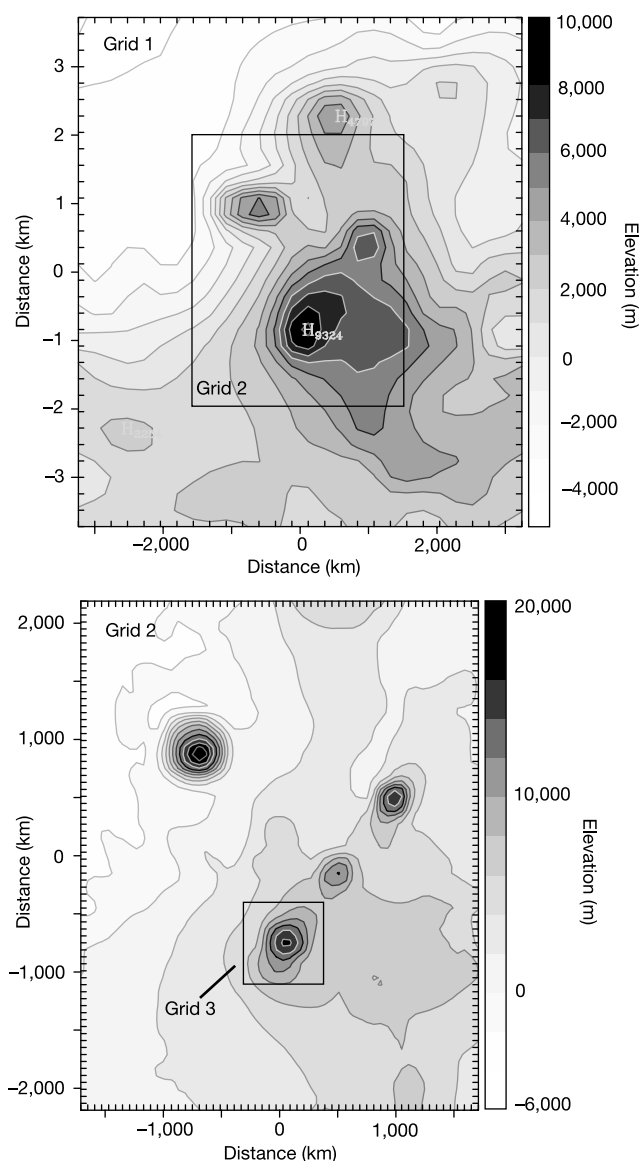


Figure 2 Topography and domain size for the first two computational grids. The horizontal grid spacing for grid 1 is 240 km, and for grid 2, 60 km. The rectangular boxes indicate the location of the next nested grid. The first grid has at least twice the topographic resolution of a typical GCM, and the Tharsis region volcanoes are only half their actual size at best. For grid 1, the minimum, maximum and contour interval for the topography is respectively 4,136 m, 9,324 m and 1,000 m. The second grid almost completely resolves the shape and height of the volcanic system. The minimum, maximum and contour interval for the topography on grid two is respectively -3,999 m, 19,550 m and 2,000 m. Grids three (rectangular box in grid two) and four (not shown) focus entirely on Arsia Mons, and allow the detailed simulation of the thermal circulation and the genesis of the spiral dust cloud. MRAMS properly represents the thermal circulations forced by the volcanoes, because the dimensions of the volcanoes are very near the actual values on the inner nested grids.

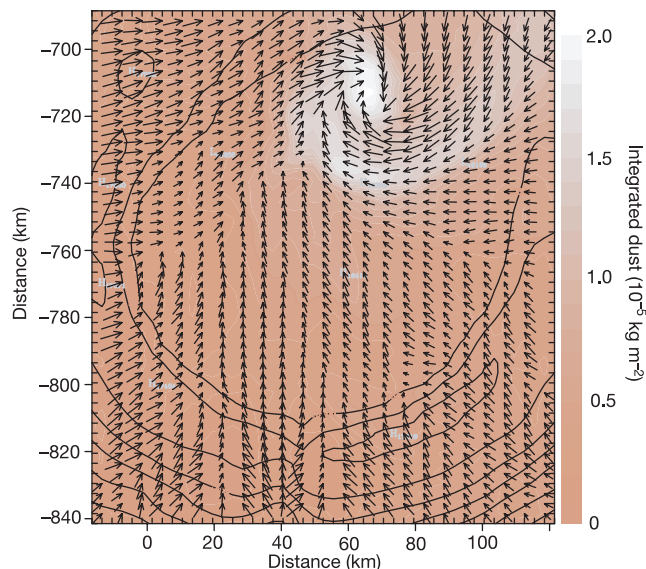


Figure 3 The spiral dust cloud simulated by the Mars Regional Atmospheric Modelling System (MRAMS), as seen on the fourth computational grid, which encompasses the caldera of Arsia Mons. Shading indicates the integrated dust mass per km^3 . Solid contours indicate topography elevation. Vectors indicate wind speed and direction. The strongest wind speed is 42 m s^{-1} . The spiral cloud feature and winds reveal a clockwise, convergent circulation, with maximum dust concentration near its centre. This figure should be compared with the imaged dust cloud in Fig. 1.

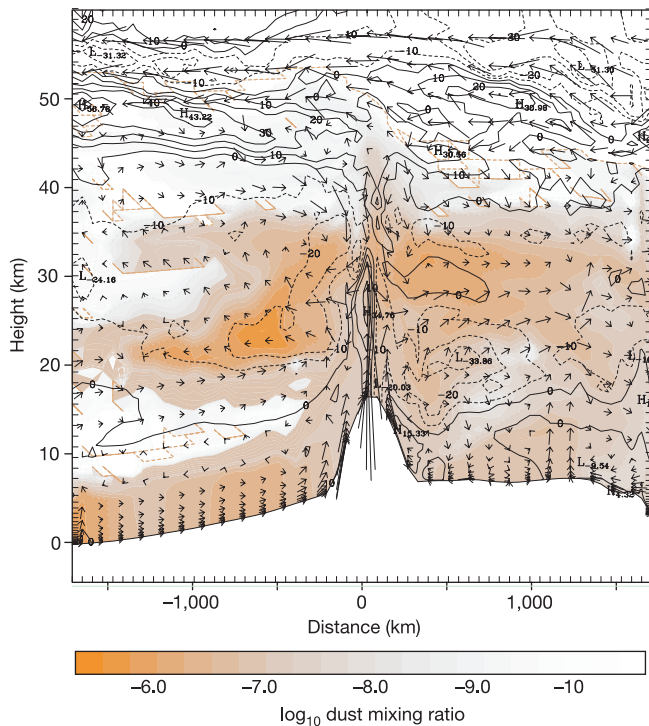


Figure 4 A vertical, east-west cross-section from grid two, cutting through the centre of Arsia Mons. Shaded regions are $\log_{10}$ of the dust mixing ratio. Contour lines indicate meridional wind: solid lines, northward-moving air (into the page); dotted lines, southward-moving air (out of the page). Vectors indicate wind velocity in the plane of the figure. The maximum horizontal wind speed is 95 m s^{-1} . The maximum vertical velocity is 9 m s^{-1} . The dust takes the form of a mushroom-shaped cloud. The upper-level outflow from the thermal circulation advects the dust more than 2,000 km downwind. The meridional wind shows a tight clockwise rotation directly above the caldera. The outflow region to either side of the volcano exhibits a weaker anticlockwise rotation.

(not shown). It would not be unexpected for a water-ice cloud to develop at the top of the thermal circulation. Such cap clouds have been observed by the MOC and by the Viking orbiter¹². But the actual amount of water vapour in the air is unknown, so we chose to run the model without the presence of any water substance in this study.

The GCMs that are typically used to study the climate of Mars have too coarse a grid spacing to properly resolve Arsia Mons (or the other volcanoes, all of which exhibit similar circulations), and are therefore unable to capture the magnitude of the thermal circulation or the dust injection mechanism associated with the circulations. These same models would not properly reproduce the radiative balance of the atmosphere, as they would not reproduce the dust loading. The easterly winds in the outflow branch (Fig. 4) effectively enhance the depth of the upper-level large-scale easterly winds. Westerly winds in the outflow located below the large-scale upper-level easterly winds increase the wind shear. Consequently, the thermal circulation creates a large perturbation in the larger-scale momentum and thermal fields.

Mars is dotted with numerous topographic features that are too small in horizontal extent to be captured by GCMs, but which may produce large-scale thermal circulations that could perturb the general circulation. Our results suggest that mesoscale thermal circulations may collectively be important in the atmospheric dust budget, and individually can produce strong regional perturbations in the background large-scale flow. □

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Cavity solitons as pixels in semiconductor microcavities

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Cavity solitons are localized intensity peaks that can form in a homogeneous background of radiation. They are generated by shining laser pulses into optical cavities that contain a nonlinear medium driven by a coherent field (holding beam). The ability to switch cavity solitons on and off^{1,2} and to control their location and motion³ by applying laser pulses makes them interesting as potential 'pixels' for reconfigurable arrays or all-optical processing units. Theoretical work on cavity solitons^{2–7} has stimulated a variety of experiments in macroscopic cavities^{8–10} and in systems with optical feedback^{11–13}. But for practical devices, it is desirable to generate cavity solitons in semiconductor structures, which

would allow fast response and miniaturization. The existence of cavity solitons in semiconductor microcavities has been predicted theoretically^{14–17}, and precursors of cavity solitons have been observed, but clear experimental realization has been hindered by boundary-dependence of the resulting optical patterns^{18,19}—cavity solitons should be self-confined. Here we demonstrate the generation of cavity solitons in vertical cavity semiconductor microresonators that are electrically pumped above transparency but slightly below lasing threshold²⁰. We show that the generated optical spots can be written, erased and manipulated as objects independent of each other and of the boundary. Numerical simulations allow for a clearer interpretation of experimental results.

Arrays of cavity solitons (CSs) are nonlinear optical structures²¹ within which independent manipulation of the intensity peaks is possible. Cavity solitons belong to the class of localized structures, which also exist in other fields (see, for example, ref. 22), and arise under conditions of coexistence of a homogeneous stationary state and a patterned stationary state for the same values of parameters. Localized structures coincide with the pattern state in a certain restricted region of the plane, and with the homogeneous state outside. A CS corresponds to a localized structure with a single peak. Once 'written' by injecting a laser pulse, the CS can be 'erased' by injecting another pulse, out of phase with respect to the holding beam, in the location where the CS lies². In the presence of a phase or intensity modulation in the holding beam, CSs tend to move to the nearest local maximum of the modulated profile³.

Our experimental set-up is schematically shown in Fig. 1. It consists of a large-area vertical cavity surface emitting laser (VCSEL, 150 μm diameter), operated as an amplifier; injected into this laser is a coherent field, generated by a high-power edge emitting laser

with an external cavity grating. Its wavelength can be continuously tuned in the range 960–980 nm. An acousto-optic modulator or a polarizer controls the external field intensity. The VCSEL is a bottom emitter²³. One of the electrodes is deposited on the top, covering the whole transverse size of the laser. A full-area n-type contact with a circular emission window is deposited at the back of the GaAs substrate. The large distance between this ring and the active medium ensures a better uniformity of the current than in the case of top-emitter lasers.

We used numerous samples, progressing in time towards optimization of the device architecture. Broad-area VCSELs usually exhibit a strong gradient of the cavity length along the transverse section, owing to the standard epitaxial growth techniques. It is important to note that the output intensity distribution of the free-running VCSEL is not strictly uniform. At currents around 180 mA, the intensity distribution can be associated with a Gauss–Laguerre TEM_{0n} mode (Fig. 2a), with n of the order of 30 or larger, which implies that the system is able to operate with a very large Fresnel number. An analysis of the spatially resolved optical spectrum (Fig. 2b) indicates that the free-running laser emits several frequencies, and that the spectral composition varies with the spatial position across the sample. We can also conclude that the centre region of the VCSEL behaves independently of the boundaries, and hence that the spatial correlation length is much smaller than the size of the sample.

Operating at current values for which the centre region is below threshold, we now inject a coherent holding field: the spatial intensity output distribution undergoes marked changes in that region, while the region near the boundary remains qualitatively unchanged. We estimate that the boundaries control the characteristics of the pattern over distances of around 10 μm . A homogeneous low-intensity region appears on the right-hand side of the sample (Fig. 2c). It is delimited by a well-defined line formed by several high-intensity spots, orthogonal to the cavity resonance gradient. On the left of that line, we see a pattern whose spatial wavelength decreases as it approaches the boundary layer. The

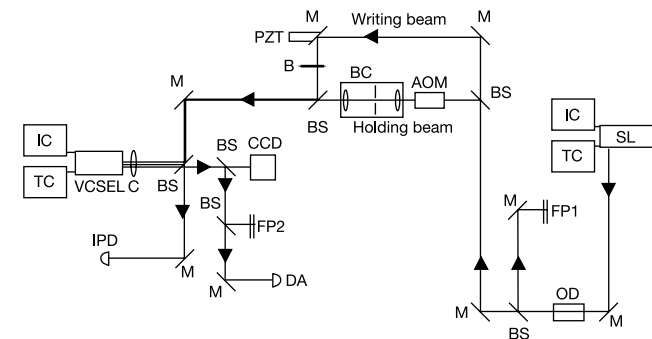


Figure 1 Schematic experimental set-up. SL, high-power edge emitter laser; its frequency is controlled by means of a grating. IC, current stabilization; the overall current is stabilized better than 1%. TC, temperature controller. A Peltier junction ensures thermal stabilization of better than 0.01 °C. OD, optical diode to isolate the laser producing the holding beam from optical feedback. FP1, FP2, Fabry–Perot resonators that measure the optical frequency of the holding and writing beams (FP1) and output beam (FP2). FP2 is coupled to a fibre in order to measure the spatially resolved optical spectra. The free spectral range (FSR) is 140 GHz, and the finesse is 100. AOM, acousto-optic modulator to change the intensity of the holding beam. BC, beam expander–configurator, used to enlarge and to conform the holding beam. VCSEL, broad-area (150 μm) vertical cavity surface emitting laser. C, collimator. CCD, charge-coupled-device camera used to monitor the time-averaged intensity distribution (near field) of the beam reflected by the VCSEL and of the holding beam. IPD, photo-detector to measure the intensity of the holding and writing beams. DA, detector array allowing simultaneous measurement of the intensity at different points of the transverse pattern. PZT, piezo-electric ceramic used to change the phase of the writing beam relative to the holding beam. B, blocker. M, mirrors. BS, beam-splitters. The transverse intensity distribution of the master oscillator is spatially filtered and shaped by lenses. Its amplitude is monitored by a photodiode and its optical frequency analysed by a Fabry–Perot interferometer with a free spectral range of 2.5 THz and a finesse of 140. An optical isolator is used to avoid feedback from the sample into the master oscillator.

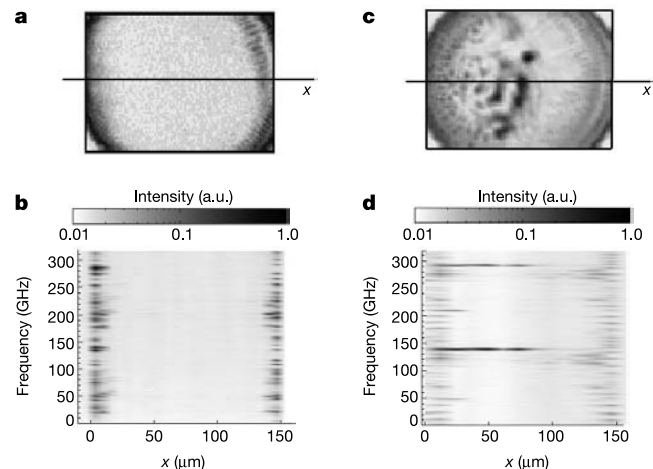


Figure 2 Average intensity distribution and spatially resolved power spectra (along the lines labelled x in **a** and **c**) for the free running laser (FRL) and for the driven VCSEL. **a, b**, The intensity distribution (**a**) and the spectrum (**b**) for the FRL pumped at 300 mA. Emission at several well-defined frequencies is observed near the boundary. **c, d**, The intensity distribution (**c**) and the spectrum (**d**) for the VCSEL with injected field. The darker lines (high intensity) in **d** correspond to the frequency of the injected field. The frequency interval between the two dark lines in **d** corresponds to the free spectral range of the Fabry–Perot resonator. The region close to the boundary still shows emission at several frequencies, while the centre region is locked at the frequency of the external field. No defined frequency is observed in the homogeneous region because the intensity is not high enough to allow a measurement with the sensitivity of our detectors.

transition from a homogeneous to a patterned region can be understood by considering that the detuning between cavity resonance and the frequency of the external field is changing along the gradient, and that pattern formation favours larger blue detunings. The spatially resolved optical spectrum (Fig. 2d) shows several frequencies close to the transverse boundary of the VCSEL. However, beyond the boundary layer, the dominant frequency becomes that of the injected field—that is, the VCSEL is locked to the master oscillator. The line separating the pattern and the homogeneous field phase can be interpreted as the locus of the spatial positions where the local values of the cavity resonance and field intensity meet the condition for the onset of a pattern-inducing modulational instability²¹.

This interpretation is supported by our theoretical analyses and simulations, which are based on the model formulated in ref. 16 and generalized to take into account the following: the spatial profile of the holding beam and of the electric current, the constant gradient in cavity resonance, and the unavoidable irregularities in the layers of the Bragg reflectors. The gradient implies that the cavity detuning parameter θ varies uniformly along the sample. Figure 3 shows that the line that marks the instability boundary corresponds precisely to the line that separates the pattern and the homogeneous region in the sample.

Fixing all parameter values (amplitude and frequency of the external field and pumping current), we inject a small focused beam (the writing beam) into the homogeneous region. Starting with no spot, the writing beam is capable of generating a high-intensity spot with a diameter of the order of $10\ \mu\text{m}$ when it is in phase with the holding beam. If we remove the writing beam, the bright spot remains 'on' indefinitely. We then change the writing beam phase by π , and inject it again into the sample where the bright spot is. The spot disappears. We remove the writing beam again, and the spatial intensity distribution becomes the same as it was at the beginning of

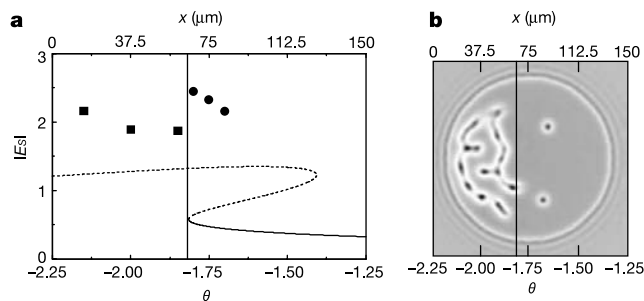


Figure 3 Numerical simulation and theoretical interpretation of the spatial field profile. **a**, The stable (solid) and unstable (broken) portions of the curve of the intracavity field versus the cavity detuning parameter θ , in the homogeneous stationary solution as proceeding from an analytical evaluation of the pattern-inducing modulational instability. It follows from a linear stability analysis of the model equations under plane wave holding field and flat current profile. Such approximations are reasonably met in the central region of the sample. The stable part of the plane-wave stationary curve terminates at $\theta = -1.81$, which corresponds to the instability boundary; it is shown by the vertical line. On the left of the line are patterns whose maximum intensities are indicated by squares. On the right, where the homogeneous background is still stable, CSs can be excited (maximum intensities marked by circles). **b**, The numerical transverse field intensity profile. The lower and upper scales indicate the value of θ that corresponds to each coordinate x . The vertical line in **b** corresponds to that in **a**; it can be seen that it satisfactorily meets the actual boundary of the patterned region. Two CSs are excited in the homogeneous region by applying two 6-ns pulses at delayed times. In the following evolution (90 ns), the two CSs move under the combined influence of the detuning gradient and the imperfections, for less than 30 ns, and are eventually pinned in the two locations (just a few micrometres away from where the switching beams were addressed). This simulation includes a time averaging comparable (1 μs) to that of the CCD used in the experiments (Fig. 4).

the experiment. This proves that we can optically manipulate a single spot, which we claim to be a cavity soliton. Injecting a writing beam in a slightly different location, we can generate a CS: but after removal of the writing beam, it is found to be at the position of the previous spot, suggesting that the latter spot is an attracting locus for CSs. This experiment was repeated at several values of the pumping current between 320 and 360 mA.

By changing the frequency of the injected field, we find various positions where a CS can be located, always to the right of the limit between the homogeneous state and the pattern. Thus, such structures exist in a relatively wide range of injected field intensity and/or pumping current, and always close to the critical detuning value corresponding to the modulational instability. The fact that only a single stable soliton location can exist for fixed parameters is attributed to the presence of the strong resonance gradient. This acts as 'wind', which tends to blow the CS (with a velocity proportional to the gradient) out of its existence region. Thus we can observe a stationary spot only if we can vectorially compensate the force exerted by the cavity length gradient by a force exerted by an intensity gradient of the holding beam, which tends to attract the CS towards the beam centre. Such a compensation can occur only in one spatial position in the transverse plane.

In order to show that more than one spot can be manipulated independently (and therefore that we can definitively identify them as cavity solitons) for a fixed set of the operational parameters, we

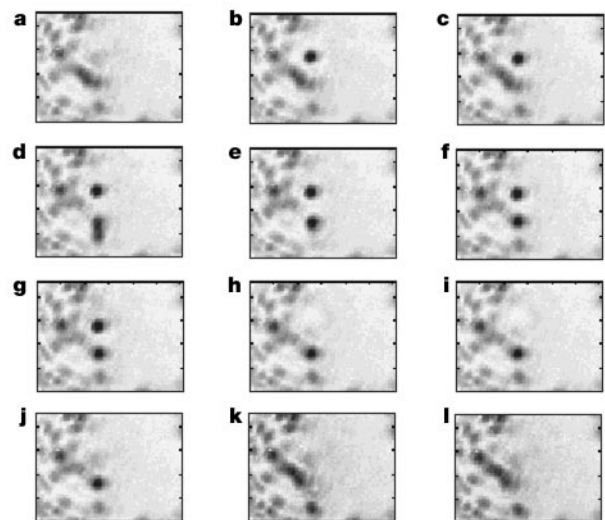


Figure 4 Experimental demonstration of independent writing and erasing of CSs. The intensity distribution of the output field is shown over a $60\ \mu\text{m} \times 60\ \mu\text{m}$ region in the sample centre. The holding beam is always on, with a waist ranging from 150 to $250\ \mu\text{m}$ with no significant effect; all other parameters are kept constant. The writing beam power is approximately $50\ \mu\text{W}$ and the holding beam power is 8 mW, although no optimization of such values have been attempted. The persistency of the excited CSs exceeds the observation time ($>1\ \text{min.}$). **a**, The writing beam (WB) is blocked. **b**, The $15\text{-}\mu\text{m}$ focused WB impinges on the homogeneous region; it induces the appearance of a single high-intensity spot (dark in the figure) in a limited region of space; **c**, the WB is blocked again, a $10\text{-}\mu\text{m}$ spot remains and is stable; **d**, the WB is displaced in position and switched on again. It generates a second spot; **e**, **f**, the WB is blocked again and the two bright spots coexist; **g**, the WB is positioned again on the first spot; **h**, the relative phase of the WB relative to the holding beam is changed by π , and the upper spot disappears; **i**, the WB is blocked again, the lower spot persists undisturbed; **j**, the WB is switched on again at the position of the lower spot; **k**, the relative phase of the WB is again brought to π and also the second spot disappears; **l**, the WB is blocked, and the intensity distribution is identical to **a**. Repeated switchings yield stable CS pairs at different locations, for current between 270 mA (sample centre below lasing threshold) and 320 mA (above threshold), thus showing that sample roughness pins the CSs but does not limit in principle the multiplicity of the structures' configurations.

designed a sample to minimize such a gradient and to maximize uniformity in parameter values across the whole section. It presents a central region where the gradient almost vanishes. The current crowding at the borders, with associated localized lasing, still exists. All other characteristics of the sample remain unchanged with respect to the first one. We repeat the experiments with the new sample, and we observe (Fig. 4a) that the homogeneous state can cover most of the diameter of the VCSEL; this occurs over an appreciable range of frequencies of the external field. A boundary is still observable between a pattern and the homogeneous state, on the left side of the sample. By injecting the writing beam inside the homogeneous state, we can generate a CS that remains when the writing beam is removed. We then apply this beam in a different location without changing any parameter value, and a second spot is generated. This spot will also persist after removal of the writing beam. We reach, then, the situation in which two CSs exist. Changing the phase of the writing beam and re-injecting it successively at the location of each spot, we erase each spot in an independent way. The full series is displayed in Fig. 4. In the experiment, the position of the CS can be changed by adding a weak gaussian beam (in phase with the holding beam and with a smaller waist) in a location lying up to three CS diameters off the soliton peak. After removal of this additional gaussian beam, the CS comes back to one of the previous positions.

The theoretical analyses and numerical simulations had the following features, yielding a consistent interpretation on the observed behaviour of CSs. When simulating the switch-on of a CS in the sample with small resonance gradient ($\nabla\theta$), the CS follows $\nabla\theta$, drifting leftwards until it merges with the pattern. The small intensity gradient of the gaussian holding beam alone is unable to counterbalance the drift. When a stochastic process simulating the sample roughness is accounted for, the CS moves to a location where it remains fixed. The conditions for such 'trapping' depend on the balance between the local resonance and $\nabla\theta$. The number and distribution of such 'trapping sites', related to the imperfections of the device, are interspersed densely enough to allow for a large number of stable CS locations throughout the sample. This effect of layer-related roughness is rather new to investigations²⁴: it does not prevent CS motion under the influence of additional field gradients, so that CSs can be manipulated exactly as in the experiment. In particular, the CS drift can be governed by injecting a second gaussian beam with waist smaller than that of the holding beam, but larger than the CS radius. When this beam is positioned close to where the CS has been turned on, it attracts the CS towards its maximum, and traps it in a location where the pull induced by $\nabla\theta$ is balanced by the intensity gradient; this occurs in a range approximately on the order of the gaussian waist, in agreement with the experiment.

Using CSs, we have realized a monolithic two-bit all-optical information processor: we consider that these results open a possible way to developing a practical device. The future development of our results requires an increase in the number of CSs that can be simultaneously present, and also a way of inducing controllable motion of CSs. To achieve these goals, it will be necessary to introduce appropriate spatial modulations in the holding beam, and to further improve the homogeneity of the sample in the transverse plane. □

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Stacking of conical molecules with a fullerene apex into polar columns in crystals and liquid crystals

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Polar liquid crystalline materials can be used in optical and electronic applications, and recent interest has turned to formation strategies that exploit the shape of polar molecules and their interactions to direct molecular alignment^{1,2}. For example, banana-shaped molecules align their molecular bent within smectic layers³, whereas conical molecules should form polar

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columnar assemblies^{4–9}. However, the flatness of the conical molecules used until now^{4–6,9} and their ability to flip^{7,8} have limited the success of this approach to making polar liquid crystalline materials. Here we show that the attachment of five aromatic groups to one pentagon of a C₆₀ fullerene molecule yields deeply conical molecules that stack into polar columnar assemblies. The stacking is driven by attractive interactions between the spherical fullerene moiety and the hollow cone formed by the five aromatic side groups of a neighbouring molecule in the same column. This packing pattern is maintained when we extend the aromatic groups by attaching flexible aliphatic chains, which yields compounds with thermotropic and lyotropic liquid crystalline properties. In contrast, the previously reported fullerene-containing liquid crystals^{10–17} all exhibit thermotropic properties only, and none of them contains the fullerene moiety as a functional part of its mesogen units. Our design strategy should be applicable to other molecules and yield a range of new polar liquid crystalline materials.

The chemical nature of side groups can tune the assembly of fullerene derivatives into a range of different nanostructures¹⁸. In our molecular design, which builds on the C₅ symmetry of the C₆₀, we attach five aromatic groups to form a conical cavity with chemical affinity for C₆₀ (Fig. 1a). The synthesis involves covalent attachment of five biphenyl groups around one pentagon of C₆₀, using an organocopper-based strategy¹⁹ that produces the pentakis(biphenyl)fullerene **1** on a 10-g scale in 99% isolated yield. The synthesis of molecules with a deep conical structure and flexible side chains started with the addition of five phenol groups to one pentagon, followed by coupling with a benzoate group bearing

two long aliphatic side chains. With this modification, the molecule acquires a deep cavity with an aromatic interior at the bottom (red/blue, Fig. 1b) and an aliphatic periphery at the top (grey, Fig. 1c). This design should favour columnar stacking of the molecules into one-dimensional arrays (Fig. 1d): the low affinity of the aliphatic side chains to C₆₀ provides a chemical driving force for microphase segregation^{2,20}. We note that C₆₀ is highly soluble in aromatic hydrocarbons while insoluble in saturated hydrocarbons²¹.

The molecules **2–5** bearing hydrocarbon side chains were synthesized in three steps: five-fold addition of 4-(THPO)C₆H₄MgBr (where THP is a tetrahydropyranyl protective group) to C₆₀ in the presence of CuBr·Me₂S and acid hydrolysis of the THP group afforded (4-HOC₆H₄)₅C₆₀H in 92% yield, followed by acylation of the resulting five phenolic groups with an appropriate acid chloride afforded **2–5** in about 60% overall yield on a multigram scale. Compounds were purified with gel permeation chromatography to analytically pure quality and used for the experiments.

Single crystals of **1** were grown in a mixture of chlorobenzene and diethyl ether. The crystal belongs to the orthorhombic crystal system and the space group of *Pbca*. A unit cell comprises four pairs of two molecules stacked in a head-to-tail manner, and infinite translation of the unit cell along the *A* axis yields one-dimensional arrays of stacked molecules (Fig. 2a). The stacking period of the molecules within a column is 11.1 Å, which includes a small void between neighbouring molecules in the same column. The columns are packed in a hexagonal manner (Fig. 2b). The packing is slightly distorted: The shorter intercolumnar distance (*a*_{hex}) is 14.2 Å

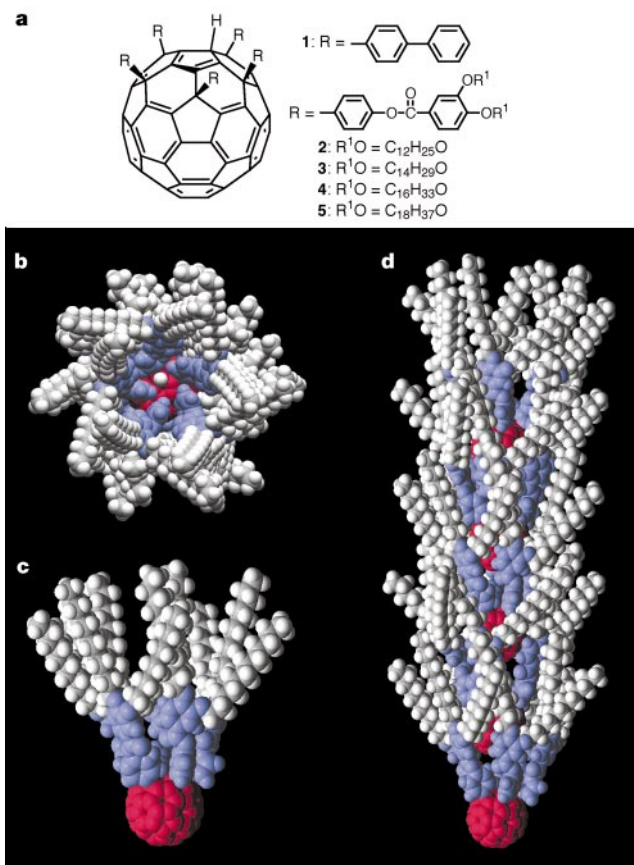


Figure 1 Fullerene derivatives with deeply conical structures. **a**, Chemical formulae of **1–5**. **b**, Top view of **2**. Colour code: red, fullerene core; blue, aromatic groups, and grey, alkyl chains. **c**, Side view of **2**. **d**, A stack of five molecules of **2** (based on molecular mechanics calculations and on the SAXD data described in the text).

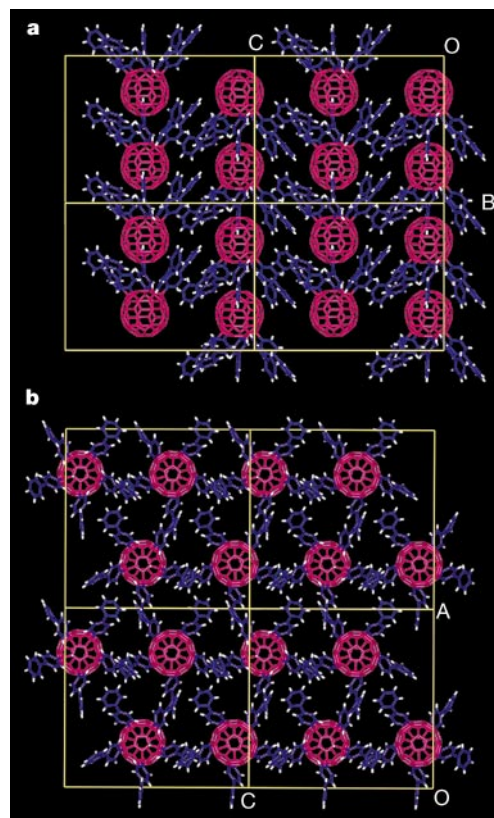


Figure 2 Crystal packing of biphenyl compound **1**. **a**, View from *A* axis and **b**, from *B* axis. For clarity, chlorobenzene and diethyl ether molecules are omitted and only a single layer of the molecules is shown. Crystal data for **1**·(C₆H₅Cl)·(Et₂O): C₁₃₀H₆₁Cl₅O₁, orthorhombic space group *Pbca* (number 61), *a* = 27.7100(9) Å, *b* = 22.1850(18) Å, *c* = 28.456(2) Å, *V* = 17493(2) Å³, *Z* = 8, *T* = 213(2) K (standard deviation in parentheses), MacScience DIP2030 diffractometer, Mo_K radiation (λ = 0.71073). The full-matrix least-square refinement with 15,517 reflections and 1,191 parameters converged to *R*1 factor = 0.1249, *wR*2 factor = 0.3481, and goodness of fit = 1.367.

(Fig. 3c) and the longer one is 17.2 Å. The fullerene core of **1** sits in the cavity created by the five biphenyl groups of the next molecule in the column. Each column is oriented in a direction opposite to the neighbouring columns, with the fullerene cores forming layers perpendicular to the column axes owing to intercolumnar biphenyl-CH/biphenyl- π interactions.

Systems containing fullerenes can exhibit liquid crystalline behaviour, but are usually thermotropic liquid crystals^{10–15}. In contrast, compounds **2–5** exhibit both thermotropic and lyotropic liquid crystalline properties. We find that these compounds display thermotropic hexagonal columnar phases up to temperatures around 140 °C (Fig. 3a). As the side chain increases in **2–5** from 12 carbons to 18, the enthalpy change of the liquid crystal-isotropic transition decreases from 21.0 to 5.0 kJ mol^{–1} whereas the glassy to liquid crystal transition temperature increases monotonously from –47 °C to –7 °C.

The small-angle X-ray diffraction (SAXD) spectrum of **2** at 60 °C shows a sharp reflection with a *d*-spacing of 31.4 Å (100), and smaller peaks associated with *d*-spacings of 16.8 (110), 15.6 (200) and 14.3 Å (001) (Fig. 4a and b). The first three peaks correspond to the reflections of a hexagonal columnar structure (reciprocal *d*-spacing of 1:√3:2) and the last peak to the stacking period within the column. In a wide-angle X-ray diffraction pattern (see Supplementary Information), a broad peak centred at 4.5 Å is seen. Its position and width are typical for molten aliphatic moieties. The

intercolumnar spacing a_{hex} is 35.3 Å (Fig. 3d), which is more than twice as large as that observed in the crystals of **1** (14.2–17.2 Å, Fig. 3c). Neither the angles nor the intensities of the reflections—*d*(100), (110), (200) and (001)—change (within ± 0.6%) at temperatures between 60 and 140 °C (Fig. 4a). The measured stacking period (14.3 Å) and the intercolumnar spacing (35.3 Å) provide strong evidence of head-to-tail stacking of **2**, which is consistent with molecular mechanics modelling (MM2*-optimized) of the stack structure (Fig. 1d).

Lyotropic mesophases form for a mixture of **2** and dodecane (Fig. 3b). At relatively small **2**/dodecane ratios (w/w), a nematic columnar phase forms. The SAXD data (Fig. 4c) at a 1:1 ratio of **2** and dodecane indicate an intercolumnar distance of about 56.3 Å (at 100 °C). In fact, the SAXD spectra at 60 and 80 °C for the lyotropic hexagonal columnar phase (Fig. 4c and d) are similar to the spectra of the thermotropic mesophase (Fig. 4a and b). Differences are the larger intercolumnar distance (39.8 Å as opposed to 35.3 Å, Fig. 3d) and the appearance of a rather sharp and strong (001) reflection corresponding to an intracolumnar repetition period of 17.5 Å (as opposed to the 14.3 Å period seen in the thermotropic liquid crystal). The relatively strong (001) reflection suggests significant modulation of the electron density along the columns as well as good positional correlation along the column long axis.

Previous fullerene-containing mesogens were built by connecting fullerene molecules to an inherently mesogenic unit^{10–15}, whereas the present design of the mesogen uses the molecular symmetry of the C₆₀ to form anisotropic ‘nano-shuttlecocks’ that stack into a one-dimensional columnar supramolecular structure. This design

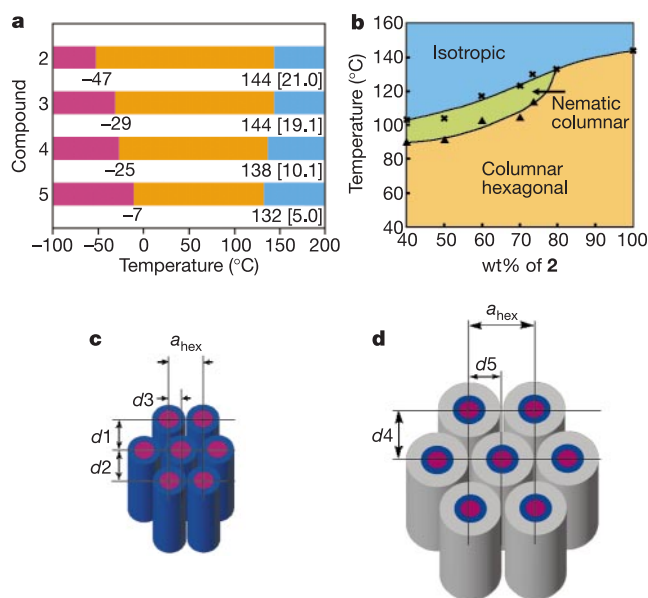


Figure 3 Crystalline and liquid crystalline properties and structures of **1–5**.

a, Thermotropic phase behaviour of **2–5**. Pink, glassy phase; orange, liquid crystalline phase; light blue, isotropic phase. Temperatures are in °C, and transition enthalpies in kJ mol^{–1} in brackets. **b**, Lyotropic phase behaviour of a mixture of **2** with various amounts of dodecane. **c**, Schematic of the crystal packing of **1**; $d_1 = 13.86$ Å, $d_2 = 13.85$ Å, $d_3 = 4.5$ Å, $a_{\text{hex}} = 14.2$ Å, stacking period is 11.1 Å (the data set refers to the shortest a_{hex} and the related parameters in this distorted hexagonal packing). **d**, Schematic of the column packing of liquid crystalline phases of **2**. For thermotropic liquid crystals of **2** at 60 °C: $d_4 = 31.4$ Å, $d_5 = 16.8$ Å, $a_{\text{hex}} = 35.3$ Å, stacking period is 14.3 Å. For lyotropic liquid crystals of a 50:50 mixture of **2** and dodecane at 60 °C: $d_4 = 34.4$ Å, $d_5 = 19.9$ Å, $a_{\text{hex}} = 39.8$ Å, stacking period is 17.5 Å. Thermal and liquid crystalline properties were determined by differential scanning calorimetry (DSC) measurements on a Mettler DSC30 system and optical observation with an Olympus BX51 microscope equipped with a Mettler FP82-HT hot stage. The liquid crystalline to isotropic transition temperature was taken at the maximum of the endothermic peak in the DSC chart on the second heating run at the rate of 20 °C min^{–1}. The glass transition temperature was read at the midpoint of the change in the heat capacity.

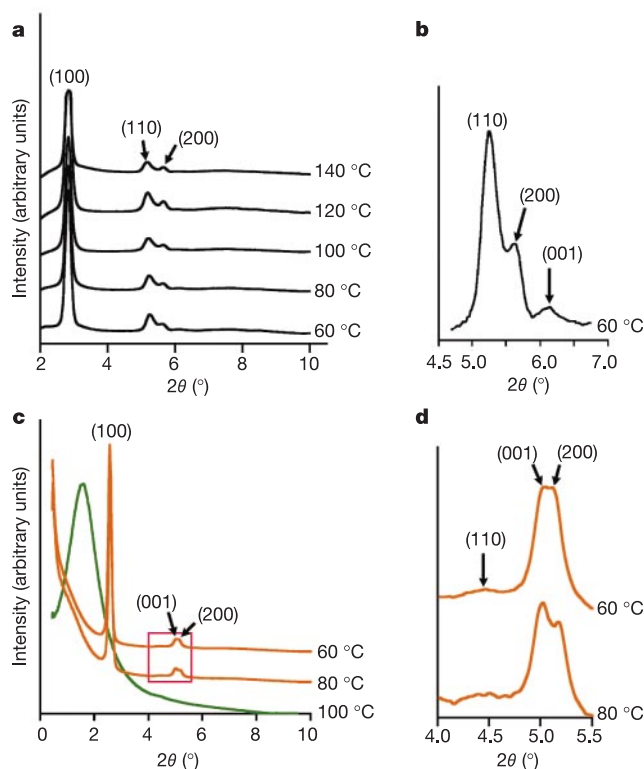


Figure 4 SAXD patterns of liquid crystals of **2–5**. **a**, The SAXD spectra of thermotropic liquid crystal of **2** at various temperatures. **b**, A more detailed view of the 4.5–7-degree region at 60 °C. **c**, The SAXD spectra of lyotropic liquid crystal of a 50:50 mixture of **2** and dodecane at 60–100 °C. The colour codes correspond to those used in Fig. 3b. The pink box region is enlarged and shown in **d**. **d**, A more detailed view of the 4–5.5-degree region at 60 and 80 °C. The SAXD profiles were recorded on a Rigaku small-angle X-ray scattering analyser by confocal multilayer mirror.

strategy should be applicable to a range of other molecules and materials. In our system, the driving force for the stacking is the aromatic/fullerene interaction in the concave cavity, and the incompatibility of the fullerene/aromatic system and the aliphatic side chain/solvent system^{2,20}. The R₅C₆₀H core structure used here exhibits electron absorption spectra and electrochemical properties similar to those of its C₆₀ parent²², and we therefore expect that the liquid crystalline material described here might exhibit interesting optical and electrochemical properties. Moreover, the species R₅C₆₀H can be transformed into a variety of stable η^5 -metal complexes R₅C₆₀M (where M is metal)²³, such as a redox-active ferrocene/fullerene hybrids²⁴; this property might allow metal-doping of the fullerene-based crystals and liquid crystals and thus expand the scope of this type of material²⁵. □

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Re–Os isotopic evidence for long-lived heterogeneity and equilibration processes in the Earth's upper mantle

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The geochemical composition of the Earth's upper mantle^{1–3} is thought to reflect 4.5 billion years of melt extraction, as well as the recycling of crustal materials. The fractionation of rhenium and osmium during partial melting in the upper mantle makes the Re–Os isotopic system well suited for tracing the extraction of melt and recycling of the resulting mid-ocean-ridge basalt³. Here we report osmium isotope compositions of more than 700 osmium-rich platinum-group element alloys derived from the upper mantle. The osmium isotopic data form a wide, essentially gaussian distribution, demonstrating that, with respect to Re–Os isotope systematics, the upper mantle is extremely heterogeneous. As depleted and enriched domains can apparently remain unequilibrated on a timescale of billions of years, effective equilibration seems to require high degrees of partial melting, such as occur under mid-ocean ridges or in back-arc settings, where percolating melts enhance the mobility of both osmium and rhenium. We infer that the gaussian shape of the osmium isotope distribution is the signature of a random mixing process between depleted and enriched domains, resulting from a 'plum pudding' distribution in the upper mantle, rather than from individual melt depletion events.

During partial melting in the upper mantle, Re is mildly incompatible, whereas Os is strongly compatible, resulting in high Re/Os elemental ratios in mid-ocean-ridge basalts (MORB) and correspondingly low Re/Os ratios in the depleted solid residue left behind³. As ¹⁸⁷Re decays to ¹⁸⁷Os with a half-life of about 42 billion years (Gyr) (ref. 4) the ¹⁸⁷Os/¹⁸⁸Os ratios of MORB and depleted mantle residue will diverge. The MORBs develop high, radiogenic ¹⁸⁷Os/¹⁸⁸Os ratios while the depleted mantle residues develop relatively low, unradiogenic ¹⁸⁷Os/¹⁸⁸Os ratios. When MORB is subducted back into the upper mantle re-equilibration with the depleted mantle residue is expected to take place, but the timescales and length scales on which this re-equilibration occurs are poorly constrained. One expectation has been that the present-day upper mantle would be characterized by a lower-than-chondritic ¹⁸⁷Os/¹⁸⁸Os ratio reflecting the time-averaged depletion in Re as a result of continuous extraction of MORB and preferential long-term storage of slabs of oceanic crust in the lower mantle. Estimates of the degree of slab isolation in the lower mantle vary widely, but are made with the assumption that the upper mantle is homogeneous with respect to its ¹⁸⁷Os/¹⁸⁸Os evolution^{5–7}. However, mounting evidence indicates that ¹⁸⁷Os/¹⁸⁸Os heterogeneities can survive on long timescales in the upper mantle^{8–12}. Examples include the discovery of ancient (0.8–1.2 Gyr) melt depletion events recorded in peridotite samples drilled from the young (~45 million years, Myr) Izu–Bonin–Mariana fore-arc subduction zone⁹ and large variations among the subchondritic ¹⁸⁷Os/¹⁸⁸Os ratios measured in peridotite samples drilled from a single section of the

Kane transform in the Atlantic Ocean¹⁰. These findings indicate that the present-day upper mantle perhaps is not homogeneous enough to be described by a single, representative $^{187}\text{Os}/^{188}\text{Os}$ ratio.

Using the Stanford/USGS Sensitive High Resolution Ion Microprobe Reverse Geometry (SHRIMP RG), we have analysed the $^{187}\text{Os}/^{188}\text{Os}$ composition of more than 700 submillimetre to millimetre-sized grains of Os-rich platinum group element alloys derived from back-arc, tectonite peridotite bodies in northwestern California and southwest Oregon¹³, USA (Fig. 1). The minerals studied are iridosmine and osmiridium that primarily consist of Os (10–95 at.%), Ir (5–90 at.%) and Ru (0–15 at.%). Rhenium concentrations are at the p.p.m. level and therefore no age correction of the measured $^{187}\text{Os}/^{188}\text{Os}$ ratios is necessary. Iridosmine and osmiridium are cumulate minerals formed by partial crystallization of basalts. They occur worldwide in association with podiform chromitites in tectonite harzburgite peridotite bodies and as detrital grains in chromite-rich placers produced by the mechanical erosion of their peridotite host rocks^{8,14,15}. The grains included in this study are solid crystals that frequently contain inclusions of chromite or have chromite attached to their surface and/or show well-developed exsolution patterns consistent with a high-temperature, that is, magmatic origin¹⁴. Iridosmine and osmiridium have previously been used to trace the Os isotopic evolution of the mantle through geological time (see, for example, refs 8 and 16). We have studied a roughly equal number of grains collected from placers at the mouth of the Rogue river (Naturhistorisches Museum in Vienna, sample A.a. 5432), from placers on the Pacific coast near Port Orford (Yale Peabody Museum, sample MIN.1.182) (Fig. 1), and from unspecified localities in northern California (Chicago Field Museum, sample M 8122); all known occurrences of iridosmine and osmiridium in northern California are found in close association with tectonite harzburgite peridotite outcrops¹⁷ (Fig. 1). Thus, the grains included in this study are derived from a large number of peridotite bodies and are expected to represent a less biased sample of the upper mantle than grains derived only from one specific peridotite source. Indeed, the three different grain samples yield similar $^{187}\text{Os}/^{188}\text{Os}$ distributions, supporting this conjecture.

The measured $^{187}\text{Os}/^{188}\text{Os}$ isotope ratios span a wide range from

extremely unradiogenic values, as low as 0.1095, to radiogenic values as high as 0.1870 (Fig. 2a). Individual grains are homogeneous with respect to their Os isotope composition⁸. This was checked by traversing (3–7 analyses) across about 50 individual grains; in each traverse all analyses yielded identical $^{187}\text{Os}/^{188}\text{Os}$ ratios within the precision of the analysis (2–4‰, $\pm 1\sigma$). The measured $^{187}\text{Os}/^{188}\text{Os}$ ratios do not correlate with Os concentration.

Figure 2b shows an expanded view of the central peak in the obtained $^{187}\text{Os}/^{188}\text{Os}$ distribution. Our data form a wide, gaussian distribution centred around 0.1245. Also shown in Fig. 2b is the compiled data set for abyssal peridotites that have been obtained over the last two decades. The abyssal peridotite data include samples from three geologically independent regions, the mid-Atlantic ridge, the southwest Indian ridge and the American–Antarctic ridge and plot under the footprint of the gaussian distribution. This is an indication that our data set is fairly representative of the upper mantle. The average $^{187}\text{Os}/^{188}\text{Os}$ value of our data set (0.1245) is lower than the currently accepted value for the primitive mantle (0.1296), which is based on Re–Os isotope systematics of spinel- and garnet-bearing xenoliths¹⁸. With the assumption of a homogeneous upper mantle this would argue in favour of long-term storage of Re in the lower mantle⁵. However, although the $^{187}\text{Os}/^{188}\text{Os}$ ratio of the present-day primitive mantle is expected to be broadly chondritic, that is, in the range 0.127–0.129, a recent re-evaluation of the xenolith data⁷ demonstrates that error propagation prevents the present-day $^{187}\text{Os}/^{188}\text{Os}$ ratio of the primitive mantle from being determined with high precision. Furthermore, as we argue below, the upper mantle is not homogeneous with respect to its Os isotope composition. These considerations make estimates of the degree to which Re might be isolated in the lower mantle ambiguous. However, our data set will offer an interesting constraint on this controversial issue when a more precise estimate for the primitive mantle becomes available.

The width and gaussian shape of the distribution in Fig. 2b has several important ramifications. First, this data set supports the emerging view that the upper mantle might be characterized by substantial, long-lived Os isotope heterogeneity^{9–12}. The least

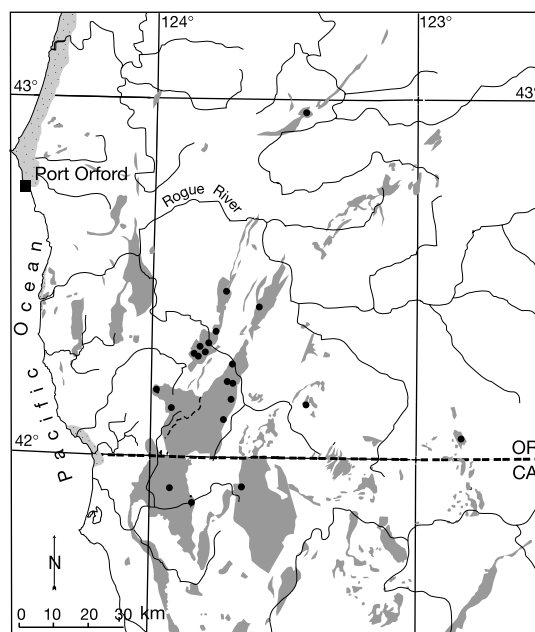


Figure 1 Map showing the distribution of ultramafic rock in southwest Oregon and northwest California and major rivers transporting chromite and platinum to the Pacific beaches. Peridotite–serpentine is shown as dark grey areas. Known occurrences of

platinoids in chromite deposits within ultramafic rock are shown as black circles²⁸. Ancient terrace deposits of black sands containing chromite and platinoids are shown as dotted, light grey areas along the Pacific coast²⁹.

radiogenic sample has a $^{187}\text{Os}/^{188}\text{Os}$ ratio of 0.1095, corresponding to a Re-depletion age³ of about 2.6 Gyr. This age provides a minimum timescale on which Os isotope heterogeneity can survive in the upper mantle, in agreement with previous observations^{9–12}. Second, because each iridosmine or osmiridium grain is homogeneous with respect to its Os isotope composition, our data provide some constraints on the minimum length scale involved in Os isotopic homogenization in the upper mantle. Assuming an Os concentration¹⁸ of 5 p.p.b. and a density of 3.3 g cm^{-3} for the upper mantle, a typical iridosmine or osmiridium grain contains Os equivalent to a mantle volume of the order of 1 m^3 . Hence, as a minimum estimate, homogenization of pre-existing Os isotopic heterogeneities has occurred on a length scale of metres. The main carrier phases for Os in the mantle are sulphide minerals^{11,12,19}. Burton *et al.*^{11,12} have demonstrated that exchange of Os by solid-state diffusion between individual sulphide inclusions hosted by mantle silicate or chromite is strongly impaired by the high (10^4 – 10^6) sulphide/silicate or sulphide/chromite partition coefficients for Os. Sulphide minerals hosted by mantle silicates can therefore remain out of Os isotopic equilibrium under mantle conditions for billions of years. Partial equilibration of Os isotopes on a length scale greater than about one metre might require partial melting to the point where sulphide inclusions are released from their host

silicate or chromite phases and dissolved into the melt. Indeed, the harzburgitic peridotite host rocks and in particular their dunite/podiform chromite regions in which the iridosmine and osmiridium grains occur *in situ*^{15,20,21} have probably experienced high degrees of partial melting and metasomatic melt-rock reactions^{22,23} that would facilitate homogenization of Os from radiogenic (MORB-rich) and unradiogenic (MORB-depleted) domains on a minimum length scale of metres. On the other hand, the Os isotope data set described here offers no constraints on the maximum length scales on which the upper mantle is heterogeneous. The width of the gaussian distribution in Fig. 2 and the low $^{187}\text{Os}/^{188}\text{Os}$ ratios of the most depleted samples (down to 0.1095) provide an indication that, in the absence of partial melting, equilibration between the radiogenic (MORB-rich) and unradiogenic (MORB-depleted) domains is inefficient. Observations of substantial Os isotope heterogeneity in abyssal peridotites on length scales of 10–100 m (refs 9, 10) might be interpreted as the characteristic length scale for upper-mantle Os isotope heterogeneity consistent with the minimum estimates made above.

We propose that the gaussian $^{187}\text{Os}/^{188}\text{Os}$ distribution in Fig. 2b is the manifestation of a process involving metasomatic melt-rock reactions that most probably took place under upper mantle conditions characterized by high degrees of partial melting, such as under a mid-ocean-ridge segment or in a back-arc setting. Generally, MORB-rich domains melt first²⁴ and the melts so produced will percolate upwards through an increasingly dense network of grain-scale pores and fractures and react with solid mantle material at shallower depths and even higher degrees of partial melting^{22–24}. Here ancient, unradiogenic Os-rich mantle sulphides encapsulated in host silicate and chromite phases^{11,12,19} are capable of equilibrating with more radiogenic Os in the melt. Thus, the gaussian distribution in Fig. 2b carries no specific age information about one or more distinct Re depletion events.

On the other hand, a gaussian distribution is the expected result of the random mixing between ancient radiogenic and unradiogenic mantle domains under conditions of relatively high degrees of partial melting²⁵. This is a consequence of the ‘central limit theorem’ according to which independent averaging of samples from any given distribution will approach a gaussian distribution with a variance that becomes smaller as the sampled volume increases^{25,26}. In terms of its Re–Os isotopic systematics, the upper mantle can therefore be envisioned as a highly heterogeneous ‘plum pudding’ mixture of these two endmembers that is sampled by the complicated processes of partial melting and infiltrative reaction. The mass balance between radiogenic and unradiogenic components determines the average $^{187}\text{Os}/^{188}\text{Os}$ of this process (that is, the peak of our gaussian distribution), which might therefore not reflect the true average $^{187}\text{Os}/^{188}\text{Os}$ ratio of the entire upper mantle.

Overall, our data lend strong support to the view that secondary metasomatic melt-rock processes define not only the major- and trace-element chemistry of mantle-derived rocks^{22,23}, but also their Re–Os isotope systematics. These processes can mask primary melt depletion features related to previous times that the material passed through an upper-mantle region with high degrees of partial melting. □

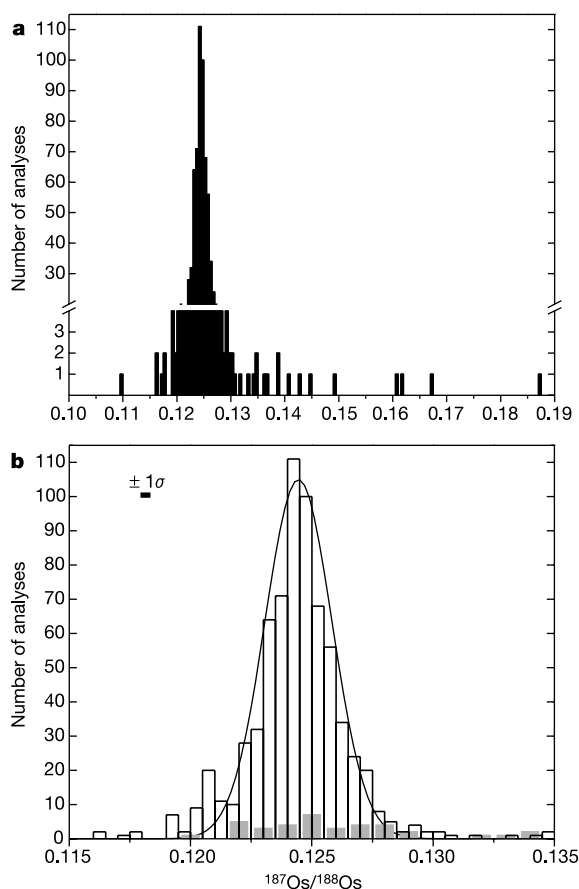


Figure 2 Histograms of Os isotope compositions of more than 700 mantle-derived iridosmine and osmiridium grains from ultramafic rock in northwest California and southwest Oregon. (See Supplementary Information.) **a**, The break in the y axis serves to increase the visibility of unradiogenic and radiogenic outliers. **b**, The histogram of the measured data points forms a gaussian distribution with a mean of around 0.1245. Solid line, fitted gaussian curve. Existing data for abyssal peridotite samples from three different oceans (compiled in refs 6 and 19) plot under the footprint of the gaussian distribution (grey columns). The $\pm 1\sigma$ error bar interval indicates the typical precision of an individual ion microprobe analysis.

Methods

The Stanford/USGS SHRIMP RG was used to measure $^{187}\text{Os}/^{188}\text{Os}$ ratios of iridosmine and osmiridium samples (see Supplementary Information) mounted in epoxy and polished to a $1\text{-}\mu\text{m}$ finish using diamond suspensions. A primary beam of O_2^- was focused to a diameter of about $30\text{ }\mu\text{m}$ on the sample with currents ranging from 20–30 nA. The magnitude of instrument mass-dependent fractionation of the SHRIMP RG was assessed by sampling an 99.999%-pure Os pellet of known Os isotope composition ($^{187}\text{Os}/^{188}\text{Os} = 0.1090081 \pm 0.0000029$) as determined by negative-ion thermal ionization mass spectroscopy (N-TIMS) at the University of Copenhagen²⁷. By measuring $^{186}\text{Os}^+$, $^{187}\text{Os}^+$, $^{188}\text{Os}^+$, $^{189}\text{Os}^+$, and $^{190}\text{Os}^+$ in an electron multiplier in peak-jumping mode the mass-dependent instrumental fractionation was found to be less than 2‰ per a.m.u. Mass 191 was monitored in order to assess the hydride contribution. Measured count rates at this mass were less than 1 c.p.s., which is insignificant in comparison with the count rates

obtained from the Os standard and the iridosmine grains, of the order of 10^5 to 8×10^5 for $^{188}\text{Os}^+$. Analysis of the iridosmine grains were performed by measuring $^{185}\text{Re}^+$, $^{187}\text{Os}^+$, $^{188}\text{Os}^+$ in an electron multiplier at a mass resolution of 6,000–7,000 in peak-jumping mode. The 99.99%-pure Os metal pellet was used as an internal standard. Typical $\pm 1\sigma$ error bar interval of individual $^{187}\text{Os}/^{188}\text{Os}$ measurement is around 3‰, similar to the external reproducibility of the standard (see Supplementary Information). $^{185}\text{Re}^+$ was measured in order to correct for the contribution of $^{187}\text{Re}^+$ to the $^{187}\text{Os}^+$ peak and, by comparison with a pure Re metal standard, estimate the Re concentration of each unknown sample.

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Linearly concatenated cyclobutane lipids form a dense bacterial membrane

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Lipid membranes are essential to the functioning of cells, enabling the existence of concentration gradients of ions and metabolites. Microbial membrane lipids can contain three-, five-, six- and even seven-membered aliphatic rings^{1–3}, but four-membered aliphatic cyclobutane rings have never been observed. Here we report the discovery of cyclobutane rings in the dominant membrane lipids of two anaerobic ammonium-oxidizing (anammox) bacteria. These lipids contain up to five linearly fused cyclobutane moieties with *cis* ring junctions. Such 'ladderane' molecules are unprecedented in nature but are known as promising building blocks in optoelectronics⁴. The ladderane lipids occur in the membrane of the anammoxosome, the dedicated intracytoplasmic compartment where anammox catabolism takes place. They give rise to an exceptionally dense membrane, a tight barrier against diffusion. We propose that such a membrane is required to maintain concentration gradients during the exceptionally slow anammox metabolism and to protect the remainder of the cell from the toxic anammox intermediates. Our results further illustrate that microbial membrane lipid structures are far more diverse than previously recognized^{5–7}.

Recently, Strous *et al.*⁸ reported the identification of a lithotroph 'missing from nature' that was capable of anaerobic ammonium oxidation. On the basis of 16S rDNA phylogeny, *Candidatus* 'Brocadia anammoxidans' and its relative *Candidatus* 'Kuenenia stuttgartiensis' were shown to be deep-branching members of the order *Planctomycetales*, one of the major, distinct divisions of the domain Bacteria^{8–10}. Anammox bacteria derive their energy from the anaerobic combination of the substrates ammonia and nitrite into dinitrogen gas. The anammox bacteria grow exceptionally slowly, dividing only once every 2–3 weeks. Anammox catabolism takes place in a separate membrane-bounded intracytoplasmic compartment, the anammoxosome¹¹. Hydrazine (N₂H₄) and hydroxylamine (NH₂OH) are the toxic intermediates, which were observed to diffuse in and out of anammox cells as free molecules^{8,12}. Indeed, the containment of these chemicals inside the anammoxosome was considered impossible, because both compounds diffuse readily through biomembranes¹³. The present discovery of the unprecedented molecular structure of the anammox membrane lipids challenges that assumption: the anammoxosome membrane is much less permeable than normal biomembranes because of the presence of unique 'ladderane' lipids.

As the starting material for the structural identification of the membrane lipids, we used a mixed bacterial culture in which 81% of

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Table 1 Composition (%) of total lipid fraction of two different strains of *Candidatus* "Brocadia anammoxidans" (strain Delft and strain Dokhaven) and the enriched anammoxosome fraction of the latter

Strain	Sample	Degree of enrichment	Characteristic fatty acids*	Lipid composition (%)		Ladderanes calculated†
				Ladderanes	Others‡	
Delft	Enrichment culture	79–83	10	58	32	70–74
	Percoll-purified cells	99.5–99.8	13	50§	37	50
Dokhaven	Enrichment culture	77–83	18	34	48	41–43
	Purified anammoxosomes	45–55	0	53	47	96–116

The Delft strain has the highest fraction of ladderane lipids.

*Iso C₁₆ and monomethyl and dimethyl branched C₁₇ fatty acids characteristic for planctomycetes (see the text).

†All other lipids (amongst others: all other fatty acids, *n*-alkanols, triterpenoids and squalene).

‡Ladderane fraction of anammox lipids calculated from the degree of enrichment and the ladderane content measured.

§Owing to the small volume of this sample, it was analysed only after direct alkaline hydrolysis of total cells. This procedure yields more nonspecific lipids (such as normal fatty acids), leading to lower relative amounts of ladderanes and masking the expected enrichment.

the population consisted of *Candidatus* 'B. anammoxidans'. To prove that the target lipids were really present in *Candidatus* 'B. anammoxidans' and not in one of the other bacteria from the culture, we subsequently identified the new lipids in a 99.5% pure suspension of *Candidatus* 'B. anammoxidans' obtained by density-gradient centrifugation⁸. We identified a variety of abundant unknown lipids in this bacterium. Two of these lipids were isolated and resolved structurally by high-field two-dimensional NMR (see Supplementary Information for details). One of these lipids (I, Fig. 1a) was identified as an *sn*-2-glycerol monoether with a C₂₀ alkyl chain containing four rings or double bonds. ¹H-NMR indicated no olefinic protons, whereas eight low-field aliphatic protons were present in the 1.8–2.7-p.p.m. range; this is unusual for a cyclic alkyl chain and is only consistent with the presence of cyclobutane rings. APT (attached proton test) and DEPT (distortionless enhancement by polarization transfer) showed that the ring system contained seven CH and five CH₂ carbons. Two-dimensional ¹H-¹H (correlated spectroscopy; COSY) and ¹H-¹³C (heteronuclear multiple bond correlation, HMBC, and heteronuclear multiple quantum correlation, HMQC) correlation indicated that the ring system (designated X; Fig. 1b) was composed of three condensed cyclobutane moieties and one cyclohexane moiety

substituted with an octyl chain, which was ether-bound at its ultimate carbon atom to the glycerol unit. The configuration of the seven stereocentres was determined by one-dimensional NOE (nuclear Overhauser effect) and NOESY (NOE spectroscopy) and by the determination of the proton-proton coupling constants.

The second isolated lipid was found to be a methyl ester of a C₂₀ fatty acid containing five rings or double bonds. ¹H-NMR again indicated that no olefinic protons were present. In this case we found 15 protons in the unusual 1.8–2.7-p.p.m. range. APT and DEPT indicated that the ring system contained nine CH and three CH₂ carbons. Two-dimensional ¹H-¹H (COSY and total correlation spectroscopy TOCSY) and ¹H-¹³C (HMBC and HMQC) correlation showed that the structure was composed of five linearly concatenated cyclobutanes substituted with a heptyl chain, which contained a methyl ester moiety at its ultimate carbon atom. The three-dimensional structure of the ring system (designated Y; Fig. 1c) was solved by NOESY and selected one-dimensional NOE experiments. All rings were found to be fused by *cis*-ring junctions, resulting in a staircase-like arrangement of the fused butane rings, defined as [5]-ladderane¹⁴. Figure 1 shows that the two ring systems (X and Y) are closely related because it only requires one additional C–C bond in the cyclohexyl ring of X to form the [5]-ladderane moiety (Y).

Lipids containing the biochemically unprecedented ladderane moieties X and Y were abundant membrane lipids of two strains of *Candidatus* 'B. anammoxidans' (that is, representing 34% and 58% of total lipids; Table 1). They occurred in a wide variety of different forms (in order of relative abundance): as fatty-acid methyl esters (II), an *sn*-2-alkyl glycerol monoether (I), alcohols (III), *sn*-1,2-dialkyl glycerol diethers (IV) and an *sn*-2-O-alkyl, *sn*-1-acyl glycerol (V). The latter two compound classes were identified by high-performance liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (HPLC-APCI-MS) and gas chromatography-mass spectrometry (GC-MS). The distribution of ring structures X and Y over the different glycerol ether lipids was not random. X was found preferentially at the *sn*-2 position, whereas Y was found only at the *sn*-1 position (Fig. 1a). Membrane lipids containing X and Y were also identified in the *Candidatus* 'K. stuttgartiensis', although in lower relative amounts. Both *Candidatus* 'K. stuttgartiensis' and 'B. anammoxidans' also contained substantial amounts of 10,14-dimethylpentadecanoic acid, and iso-, *n*-, 9- and 10-methyl-hexadecanoic acids (Table 1). The last of these has been reported to be characteristic for planctomycetes¹⁵.

Apart from the ladderane moieties, the presence of the ether linkages (linking the lipids to the glycerol backbone) was also unexpected in members of the domain Bacteria but was consistent with previous results¹⁶. Ether linkages were once thought to be distinctive for the domain Archaea¹⁷. But although glycerol diethers are rare in bacteria, they have been detected in thermophiles^{18–20} and in sulphate-reducing bacteria of a microbial consortium capable of anaerobically oxidizing methane⁶. Mixed glycerol ethers/esters were found in deep-branching thermophilic bacteria such as *Aquifex* and

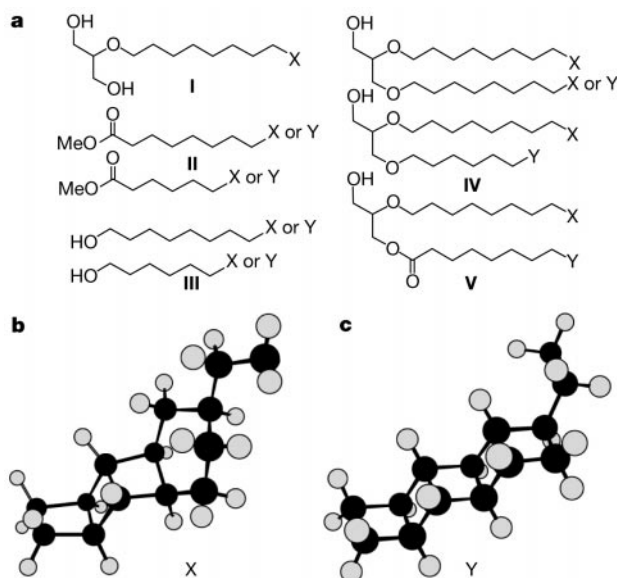


Figure 1 Structures of the anammox ladderane membrane lipids. **a**, General chemical structures of unique membrane lipids of anammox bacteria. **b**, **c**, Three-dimensional representation of ring structures X and Y. Carbon and hydrogen atoms are represented by black and grey balls, respectively. In **b** and **c** the first two carbon atoms of the side chains of these ring structures are indicated for reference. The structural identifications are based on high-field NMR determination (see Supplementary Information for details) and quantum-chemical simulations with density functional theory (B3LYP/6-31G**).

Thermotoga^{20–22} and, recently, in two mesophilic sulphate-reducing bacteria²³.

The structures of the ladderane membrane lipids are unique in nature. Functionalized ladderanes have been prepared synthetically and hold great promise as spacers and molecular optoelectronic devices; the ladderane moiety provides a rigid framework that can be appended with functional groups^{4,24}. Ladderane membrane lipids have so far been found only in anammox bacteria and in biomass of anaerobic wastewater plants in which anammox bacteria form a substantial part of the mixed bacterial population, but not in phylogenetically related planctomycetes such as *Pirellula marina* and *Gemmata obscuriglobus* (J.S.S.D., W.I.C.R. and M.S., unpublished observations). This immediately raises the question of the functional significance of the ladderane lipids. The cyclobutane ring is already quite strained and this certainly holds for the [5]-ladderane moiety composed of five linearly concatenated cyclobutane rings. Biosynthesis of such biomolecules will require a unique set of enzymes and might have a high energetic cost.

Anammox bacteria are grown in the laboratory at growth rates of less than one cell division per two weeks^{10,25}. The cells are small

(diameter <1 μm) and have the high surface-to-volume ratio characteristic of prokaryotes. Being autotrophs, they depend on an electrochemical ion gradient across a membrane for the generation of ATP. Because catabolism is slow, only few protons are translocated per unit of time, whereas the dissipation of the resulting electrochemical gradient by passive diffusion is independent of growth rate and proceeds at normal speed. Furthermore, the anammox intermediates hydrazine and hydroxylamine readily diffuse through biomembranes. From a bioenergetics perspective, the energy loss associated with the loss of one molecule of hydrazine from the anammox cells is equivalent to at least 10 full catabolic cycles, leading to a 50% decrease in biomass yield at 10% hydrazine loss. For these reasons, the limitation of diffusion is extremely

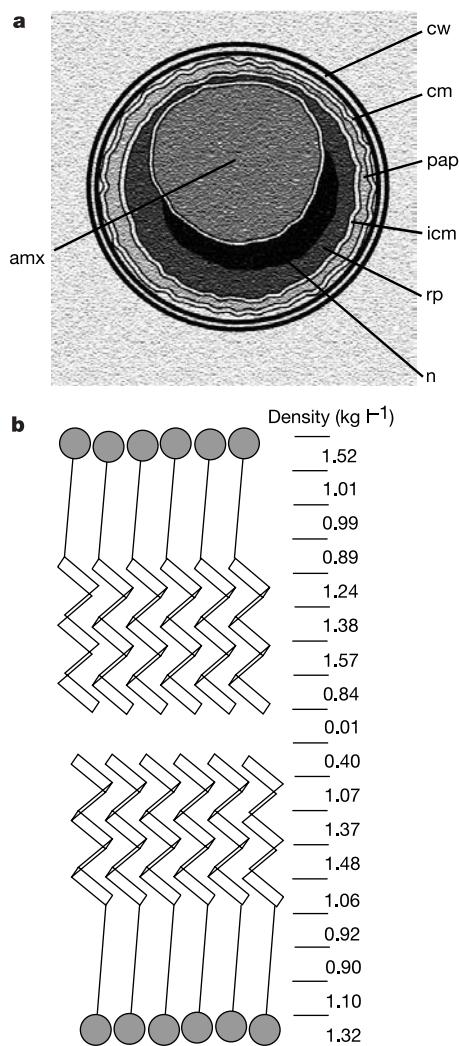


Figure 2 Model of the anammox cell and its anammoxosome membrane. **a**, Schematic representation of anammox cells (cw, cell wall; cm, cytoplasmic membrane; pap, paryphoplasm; icm, intracytoplasmic membrane; rp, riboplasm; n, nucleoid; amx, anammoxosome). **b**, Conceptual model of stacking of ladderane membrane lipids. The numbers show the density of a lipid bilayer consisting of lipid V (Fig. 1) at 298 K, as determined by molecular modelling. Conventional membranes have a density of $\sim 1.0 \text{ kg dm}^{-3}$.

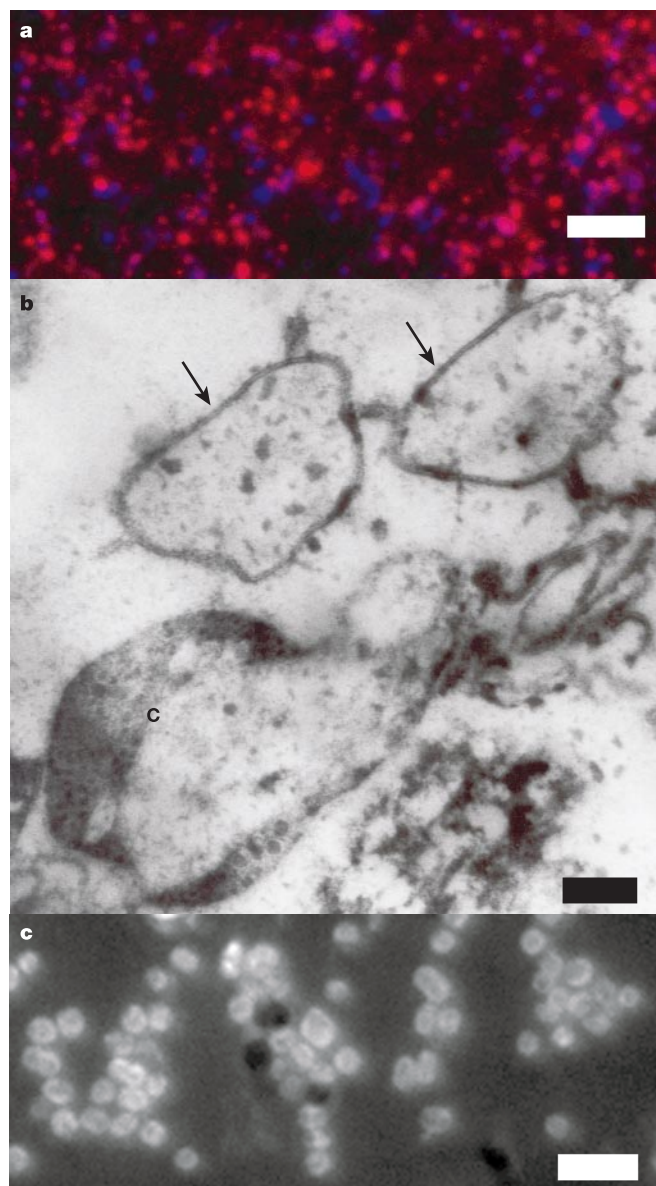


Figure 3 Microscopy of anammoxosomes. **a**, Immunofluorescence and DAPI staining of enriched anammoxosomes from *Candidatus* 'B. anammoxidans'. Anammoxosomes are stained by the polyclonal antibody rabbit anti-HAO²⁷ and the Cy-3-labelled secondary antibody sheep anti-rabbit IgG (red), but not by DAPI (blue). Incompletely lysed cells are stained by both fluorophores. Scale bar, 10 μm . **b**, Electron micrograph of two free anammoxosomes (arrows) adjacent to an incompletely lysed cell (C). Scale bar, 100 nm. **c**, Penetration of the fluorophore DiBac(4)3 into cells of *Candidatus* 'B. anammoxidans'. Cells are visible as circles owing to the unstained anammoxosome (see also Fig. 2a). Scale bar, 3 μm .

important for these bacteria. Anammox catabolism takes place inside an intracytoplasmic compartment, the anammoxosome, bounded by a separate lipid membrane (Fig. 2a). The presence of a dedicated biomembrane, more resistant to diffusion owing to its dense and rigid ladderane lipids, might be a specific adaptation to an unusual metabolism.

To compare the permeability of the different anammox membranes experimentally, we incubated the cells with 11 different fluorophores, after 6 different membrane-permeating treatments, at pH 4–9, and over a temperature range of 0–92 °C. Because lipid bilayers are fluid, a difference in permeability to fluorophores would also mean a difference in permeability to a smaller molecule such as hydrazine. Differences in permeability were revealed by epifluorescence microscopy of the cells. It seemed that the cytoplasmic membrane was relatively permeable (all fluorophores except the fluoresceins permeated without pretreatment) and stained the riboplasm homogeneously, as shown by overlapping 4,6-diamidino-2-phenylindole (DAPI) and fluorophore stains, whereas the anammoxosome was surrounded by a membrane impermeable to these fluorophores except after complete dehydration of the cells in 100% ethanol or after drying. Without such harsh treatments, even after heating the cells at 92 °C for 90 min, the anammoxosome remained unstained (Fig. 3), indicating that the membranes of the anammoxosome are unusually dense. The unusual density was confirmed by molecular modelling of a lipid bilayer composed of ladderane lipids. Its density was calculated (Fig. 2b) and the density of the ladderane part of the membrane (up to 1.5 kg dm^{-3}) was found to be significantly higher than for a conventional membrane (at most 1.2 kg dm^{-3}). Because the model consisted of only one type of ladderane lipid (V), the packing of the model membrane was probably still suboptimal compared with an *in vivo* ladderane membrane, which is much more complex with many different lipids (see Fig. 1a).

To demonstrate the presence of the ladderane lipids in the anammoxosome membrane, we enriched intact anammoxosomes from cells of *Candidatus* 'B. anammoxidans'. Microscopy revealed that intact anammoxosomes could indeed be produced (Fig. 3a, b), although incompletely lysed cells were also still present (about 50%). However, lipid analysis showed a strong enrichment in ladderane lipids in the enriched anammoxosomes fraction (Table 1). Our data show that the ladderane lipids form a substantial part of the anammoxosome membrane, that the special chemistry of these ladderanes makes this membrane denser than conventional membranes, and that such a membrane could contain the toxic anammox intermediates (for example, hydrazine) in the anammoxosome and help to maintain concentration gradients even during the slow anammox metabolism.

The identification of the anammox-specific ladderane lipids has, apart from their biochemical implications, two potential applications. First, they could be used, in addition to anammox-specific 16S rRNA gene sequences, as signature molecules for past and present anammox activity in natural ecosystems such as stratified marine waters (for example, the Black Sea) and marine sediments²⁶. Second, the study of enzymatic biosynthesis of ladderane lipids could guide organic chemists in a more controlled synthesis of ladderane derivatives with potential future applications in industry. □

Methods

Lipid analysis

Cells and enrichment fractions were extracted ultrasonically with methanol (once): methanol/dichloromethane (DCM) (once): and DCM (three times). The lipids were analysed by GC–MS after appropriate derivatization and HPLC–APCI–MS. For isolation of lipids the extract of a large batch of *Candidatus* 'B. anammoxidans' (strain Delft) was separated by column chromatography (Al_2O_3) by elution with solvents of increasing polarity. Glycerol monoether I was obtained in pure form in the most polar fraction, whereas preparative HPLC was performed on the methyl ester fraction to isolate the ladderane fatty-acid methyl esters. Isolated lipids were studied by one- and two-dimensional high-field (400–750 MHz) ^1H - and ^{13}C -NMR spectroscopy.

Isolation of anammoxosomes

Ten ml (1 g of protein per ml) of 80% enrichment cultures of *Candidatus* 'B. anammoxidans' strain Dokhaven were harvested from modified anammox sequencing batch reactors²⁵. Single cells of strain Dokhaven were produced as described previously⁸. The cells were incubated at 4 °C in 50 mM Tris–HCl buffer pH 7, containing 60 mM EDTA, 0.25 M sucrose and 5 mg ml^{-1} lysozyme. After 20 min the mixture was sonicated for 5 s and centrifuged for 5 min (10,000g); the supernatant was discarded. The resulting pellet was analysed by immunofluorescence and transmission electron microscopy. For immunofluorescence, paraformaldehyde-fixed anammoxosomes were resuspended in PBS (65 mM NaCl, 5 mM NaH_2PO_4 , pH 7.2) and immunofluorescence was performed according to standard procedures with rabbit anti-anammox-hydroxylamine oxidoreductase (HAO)^{11,27} as the primary antibody, Cy-3-labelled sheep anti-rabbit IgG as the secondary antibody, and rabbit antibodies against *Alcaligenes faecalis* as the negative control. Samples were counterstained with DAPI. For electron microscopy, anammoxosomes were fixed in 1% OsO_4 and 2% glutaraldehyde, embedded in spurr resin, thin-sectioned, and post-stained with uranyl acetate and lead citrate. Sections were inspected on a JEOL Jem-100 CXII electron microscope.

Molecular modelling

A series of NPT (constant pressure and temperature) molecular dynamics simulations were performed on periodic systems containing 3×3 lipid monomers, making up a lipid bilayer of the ladderane (composed of V) and conventional 1,2-di(octadecanoyl)glycerol lipid membranes with the use of a previously described force field²⁸. After equilibration at 25 or 298 K and 0.1 MPa with the Berendsen method²⁹ to control temperature and pressure, the nonpolar region was divided into eight partitions of equal volume and a cross-membrane density profile was determined for these partitions.

Permeation of anammox cells with fluorophores

Ten ml (1 g of protein per ml) of 80% enrichment cultures of *Candidatus* 'B. anammoxidans' strain Dokhaven or *Candidatus* 'K. stuttgartiensis' were harvested from modified anammox sequencing batch reactors²⁵. Single cells of *Candidatus* 'B. anammoxidans' strain Dokhaven were produced as described previously⁸. They were washed in 20 mM phosphate or 100 mM Tris–HCl buffers (pH 4.5, 6, 7, 8 and 9), incubated in an oven or on ice (at 0, 20, 46, 65, 80 and 92 °C), with a fluorophore (one of rhodamine 123, rhodamine B, rhodamine 6G, sodium fluorescein, dichlorofluorescein, DiBac(4)3 (Molecular Probes), fuchsin, dansyl chloride, thionine, methylene blue or benzofurazran; each at $50 \mu\text{g ml}^{-1}$ to 0.5 mg ml^{-1}), with and without a detergent (SDS, Tween 20, Triton X-100 or CHAPS) at 0.025%, with and without the addition of 1.5% chloroform or toluene, and with the addition of 30% methanol. Alternatively, the fluorophores were added after dehydration of the cells by drying at 46 °C or by stepwise dehydration in ethanol (50%, 80% and 95%) or mild sonication (5 s, 150 W, 9.5 mm tip diameter). The bacterial nucleoid was stained with DAPI (1 mg ml^{-1}). Cells were observed at 1,000× magnification with a Zeiss Axioplan 2 imaging epifluorescence microscope with filter sets 02, mf20, 24 and 26, and chromafilter set special FITC, equipped with an Axiocam black-and-white camera. Cells of *Saccharomyces cerevisiae* were used as the control, to verify the penetration of the dyes into intracytoplasmic compartments with 'usual' membranes.

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Jasmonate and salicylate induce expression of herbivore cytochrome P450 genes

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Jasmonate and salicylate are plant-produced signals that activate plant defence genes after herbivory^{1–3} or pathogen⁴ attack. Amplification of these signals, evoked by either enemy attack or experimental manipulation, leads to an increase in the synthesis of toxic compounds (allelochemicals)^{5–8} and defence proteins^{6,9,10} in the plants. Although the jasmonate and salicylate signal cascades activate different sets of plant defence genes¹⁰, or even act antagonistically^{11,12}, there is substantial communication between the pathways^{2,3,13}. Jasmonate and salicylate also contribute to protecting plants against herbivores by causing plants that experience insect damage to increase their production of volatile molecules that attract natural enemies of herbivorous insects¹⁴.

In response to plant defences, herbivores increase their production of enzymes that detoxify allelochemicals, including cytochrome P450s (refs 15, 16). But herbivores are potentially vulnerable to toxic allelochemicals in the duration between ingesting toxins and induction of detoxification systems. Here we show that the corn earworm *Helicoverpa zea* uses jasmonate and salicylate to activate four of its cytochrome P450 genes that are associated with detoxification either before or concomitantly with the biosynthesis of allelochemicals. This ability to 'eavesdrop' on plant defence signals protects *H. zea* against toxins produced by host plants.

The corn earworm, *H. zea*, is broadly polyphagous, with over 100 known host plants including herbs, shrubs and other low-lying vegetation. We chose *H. zea* to address the general issue of whether herbivorous insects can activate their enzymes that metabolize

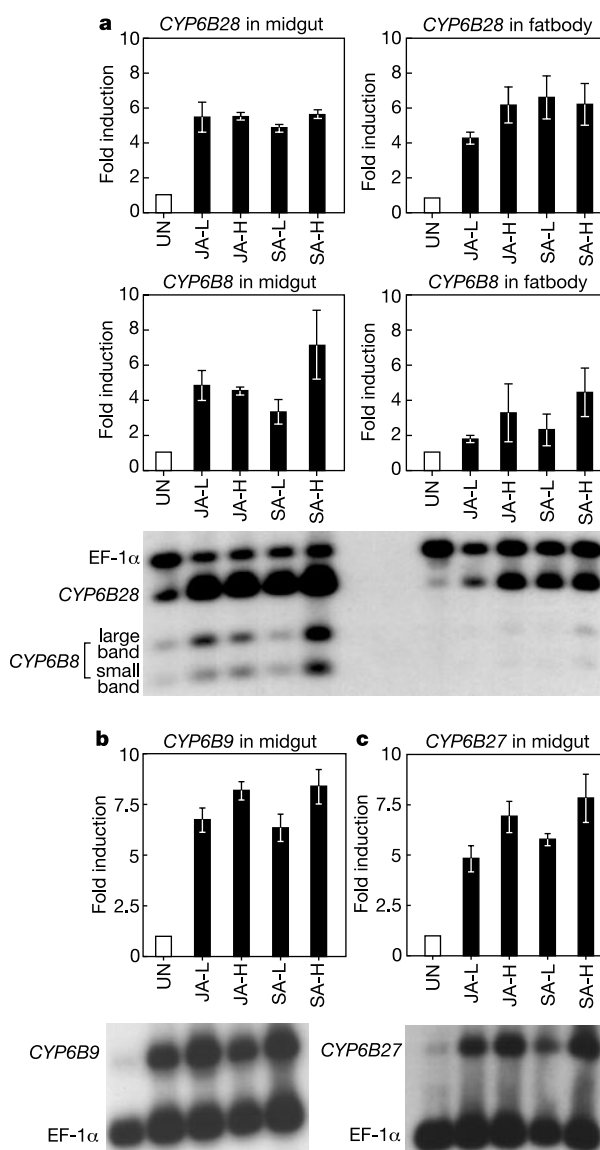


Figure 1 CYP6B gene expression in *H. zea* in response to jasmonate (JA) and salicylate (SA). **a**, CYP6B28; **b**, CYP6B8; **c**, CYP6B9; **d**, CYP6B27. Total midgut or fatbody RNAs from five different diet treatments (UN, control diet; JA-L, 2.9 $\mu\text{g g}^{-1}$ JA; JA-H, 290 $\mu\text{g g}^{-1}$ JA; SA-L, 12 $\mu\text{g g}^{-1}$ SA; SA-H, 1.2 mg g^{-1} SA) were separately amplified by RT-PCR and analysed as described in Methods. The average fold induction and standard deviations (error bars) for three independent RT-PCR amplifications are shown in the histograms; an autoradiogram of a representative blot is shown below.

Table 1 Effects of jasmonate and salicylate exposure on growth rate, weight gain and mortality of *H. zea*

Treatments	Duration of fourth instar (d)	Duration of fifth instar (d)	Mortality on day 3 (%)	Weight gain on day 3 (mg)	Pupal weight (mg)	Final mortality (%)	Final pupation rate (%)
No exposure							
Control diets	1.5 ± 0.5 d	5.5 ± 0.5 e	0.0 ± 0.0 b	384.4 ± 25.6 a	346.4 ± 13.6 a	15.6 ± 5.1 d	84.3 ± 15.1 abc
Xanthotoxin diet	3.8 ± 0.6 b	11.7 ± 0.3 b	8.9 ± 1.9 b	102.2 ± 28.3 bc	225.3 ± 16.5 b	30.0 ± 10.0 bcd	70.0 ± 10.0 cd
Celery leaves	5.7 ± 0.3 a	14.7 ± 0.6 a	33.3 ± 15.3 a	47.6 ± 2.7 d	183.2 ± 23.4 c	86.7 ± 11.6 a	12.8 ± 6.3 e
JA-L							
Control diets	1.5 ± 0.5 d	5.5 ± 0.5 e	0.0 ± 0.0 b	396.1 ± 7.8 a	348.2 ± 24.3 a	6.7 ± 5.7 d	93.3 ± 5.8 a
Xanthotoxin diet	2.5 ± 0.5 c	9.5 ± 0.5 c	0.0 ± 0.0 b	78.9 ± 15.4 bcd	226.6 ± 2.5 b	26.7 ± 11.6 bcd	73.3 ± 11.6 bcd
Celery leaves	2.5 ± 0.5 c	8.5 ± 0.5 d	3.0 ± 5.3 b	84.5 ± 17.9 bcd	178.1 ± 39.5 c	48.3 ± 16.1 bc	48.7 ± 20.1 d
SA-L							
Control diets	1.5 ± 0.5 d	4.7 ± 0.6 e	3.3 ± 5.8 b	382.1 ± 50.3 a	372.9 ± 11.6 a	10.0 ± 10.0 d	89.9 ± 10.0 ab
Xanthotoxin diet	2.5 ± 0.5 c	9.7 ± 0.6 c	0.0 ± 0.0 b	113.1 ± 9.3 b	232.7 ± 6.5 b	23.3 ± 5.8 cd	76.7 ± 5.8 bcd
Celery leaves	2.5 ± 0.5 c	10.8 ± 0.3 b	7.0 ± 6.1 b	69.9 ± 15.5 cd	166.1 ± 15.6 c	49.9 ± 10.0 b	50.0 ± 10.0 d

Effects were measured from fourth instar to pupae on celery leaves and on control and 0.5% xanthotoxin diets. The diets contained 2.9 µg g⁻¹ JA (JA-L) or 12 µg g⁻¹ SA (SA-L). Data as percentages were arcsine transformed before analysis. Results are the untransformed means ± s.d. In each column, means followed by different letters are significantly different ($P < 0.05$, modified LSD t -test).

allelochemicals in response to the plant signal molecules jasmonate and salicylate before the accumulation of plant defence compounds. Transcripts of four *H. zea* cytochrome P450 (P450) genes are inducible by furanocoumarins, chlorogenic acid, indole-3-carbinol and flavone^{16,17}, which suggests that these genes are involved in detoxifying a range of plant allelochemicals. But these four P450 genes are not universally inducible by all allelochemicals of host plants; for example, gossypol, quercetin and rutin do not induce their transcription¹⁷. Baculovirus-mediated expression of one of these proteins, CYP6B8, has shown that this protein can metabolize xanthotoxin (221.1 pmol per ml of baculovirus-expressing cell culture per min; unpublished data)—a furanocoumarin that is present in many host plants of *H. zea* and whose biosynthesis is stimulated by jasmonate and methyl jasmonate⁷.

In *Apium graveolens* (celery)^{7,18}, a host plant of *H. zea*¹⁹, xanthotoxin and the related furanocoumarin bergapten begin to accumulate after 24 h and reach maximal concentrations (representing a 40–70-fold increase) 4–6 d after the application of jasmonate and methyl jasmonate¹⁸. Accordingly, we fed fifth instars of *H. zea* for 48 h with either artificial diets supplemented with jasmonate and salicylate (at two concentrations for each chemical) or control diets, and then examined expression of four P450 genes, CYP6B8

(AF102263), CYP6B9 (AF140278), CYP6B27 (AF285829) and CYP6B28 (AF285186), in the midgut and fatbody—the principal sites of allelochemical detoxification in this species¹⁷.

Among the P450 transcripts examined, CYP6B8 and CYP6B28 (99% amino acid identity), a pair of highly conserved paralogues²⁰, were simultaneously amplified by polymerase chain reaction with reverse transcription (RT-PCR), differentiated by digestion with *Xmn*I, and quantified by gel blot analysis (Fig. 1a). In midguts, CYP6B28 transcripts were induced about 5.0-fold by jasmonate and salicylate irrespective of the concentration, whereas CYP6B8 transcripts were induced about 4.5-fold by either concentration of jasmonate, 3.3-fold by the low concentration of salicylate and 7.1-fold by the high concentration of salicylate (Fig. 1a). In fatbody, CYP6B28 transcripts were induced about 6.0-fold by either concentration of salicylate, 4.2-fold by the low concentration of jasmonate, and 6.2-fold by the high concentration of jasmonate; transcripts of CYP6B8 were increased to a lesser extent by jasmonate and salicylate (Fig. 1a).

The more divergent CYP6B9 and CYP6B27 transcripts derived from another pair of paralogous P450 genes (87% amino acid identity with CYP6B8), whose expression is restricted to midguts¹⁷, were separately detected by RT-PCR gel blot analysis (Fig. 1b, c). In midguts, CYP6B9 transcripts were induced 6.0-fold by low concentrations and 8.0-fold by high concentrations of jasmonate and salicylate (Fig. 1b), whereas CYP6B27 transcripts were induced 4.8-fold and 5.8-fold by low concentrations of jasmonate and salicylate, respectively, and 6.9-fold and 7.8-fold by high concentrations of jasmonate and salicylate, respectively (Fig. 1c). These results show clearly that expression of CYP6B is activated in the midgut and fatbody of *H. zea* at the low concentrations of jasmonate and salicylate that are associated with pest damage and allelochemical induction in its host plants^{4,21}.

To assess the specificity of this induction response, we tested further the induction of these P450 genes in response to two salicylate-related chemicals at equivalent concentrations to high concentration of salicylate. Methylparaben, which differs from salicylate in the position of its hydroxy group and in having an additional methyl ester group, did not induce any of the CYP6B genes examined. Not surprisingly, *p*-hydroxybenzoic acid, which differs from salicylate only in the position of its hydroxy group, acted as a weaker inducer than salicylate and increased the amounts of CYP6B8 and CYP6B28 transcripts roughly 2.0-fold, and the amounts of CYP6B9 and CYP6B27 transcripts 4.0–5.0-fold (Fig. 2). These results indicate that the degree of activation of *H. zea* CYP6B genes by salicylate, jasmonate and related compounds is dependent on structural features of these signal molecules.

To test whether an increase in endogenous amounts of signal substances in plants that occurs before allelochemical biosynthesis is sufficient to induce transcriptional expression, we allowed starved fourth instars of *H. zea* to damage celery leaves and then determined the ability of these leaves to activate transcription of CYP6B in a

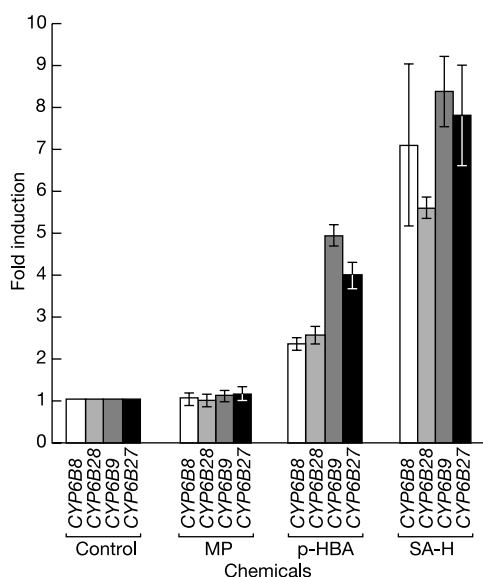


Figure 2 CYP6B expression in response to salicylate (SA) and SA-related *p*-hydroxybenzoic acid and methylparaben. Total RNAs from caterpillars fed on diets containing 1.2 mg g⁻¹ SA (SA-H), 1.2 mg g⁻¹ methylparaben (MP) or 1.2 mg g⁻¹ *p*-hydroxybenzoic acid (p-HBA) were amplified by RT-PCR and analysed as described in Methods. The relative induction and standard deviations for three independent RT-PCR amplifications are shown.

second set of fifth instars 2 and 4 h after damage. Compared with leaves from two undamaged control plants, which did not induce *CYP6B* expression, leaves from all of the plants that had been attacked for 2 and 4 h induced expression of *CYP6B28*, *CYP6B9* and *CYP6B27* (Fig. 3). Analysis of these plants indicated that, in a background of up to twofold constitutive differences in furanocoumarin content and composition among the test plants, no induced accumulation of furanocoumarin occurred. The differences in *CYP6B* expression were not correlated with variations in furanocoumarin between plants (Fig. 3), which indicated that the activation of *CYP6B* transcription resulted from an induction of jasmonate and/or other signal substances caused by the feeding behaviours of the first set of larvae. These data provide direct evidence that *H. zea* can intercept the plant defence signals elicited by its own feeding activity.

To determine whether activation of P450 genes in advance of exposure to furanocoumarins confers protection on *H. zea*, we compared the survival and growth of fourth instars that had prior exposure to low concentrations of jasmonate and salicylate for 12 h on celery leaves, 0.5% xanthotoxin diets, or control diets against that of control larvae that had not been exposed in advance to signal substances. Two-way analysis of variance (ANOVA) and multiple comparison tests on mortality, weight gain, growth rate and pupation success (Table 1) indicated that caterpillars exposed to jasmonate and salicylate survived better on celery leaves and 0.5% xanthotoxin diets for all parameters. On control diets, however, there were no significant differences in all parameters among the three treatments. These results suggest that the 'signal-eavesdropping' capability provides *H. zea* with prophylactic protection against plant defences at no additional cost to fitness in the absence of plant defences.

Reciprocal phenotypic responses characterize many antagonistic ecological interactions; if such reciprocal phenotypic change results from adaptive plasticity in the interacting species, then coevolutionary interactions may result in the evolution not only of fixed adaptations but also of phenotypic plasticity²². The induction of P450 counterdefence genes in herbivores in response to plant signal substances that are themselves inducible by herbivore damage might be an example of such phenotypic plasticity. Although it is well known that herbivorous insects can enhance the expression of detoxification enzymes (counterdefences) in the presence of plant allelochemicals (plant defences)^{15–17,23,24}, we have shown here that *H. zea* responses to plant damage are more sophisticated than was thought previously. By responding to plant signal molecules as well as the end-product allelochemicals, insects have the capacity to equip themselves before (or concomitant with) the accumulation of toxic concentrations of plant defence compounds. Although several examples have been found of plants using insect-derived signal substances to regulate their defence pathways^{1–3}, this represents to our knowledge the first example of the use by insects of plant signal molecules to regulate their defence systems against plant allelochemicals.

The ability to use plant signal molecules as cues for activating a detoxification system may be of particular value to a broadly polyphagous herbivore such as *H. zea*. In contrast to oligophagous species, which encounter a relatively narrow and generally predictable range of plant allelochemicals, generalized herbivores may encounter any of several biosynthetically distinct compounds depending on host plant choice²⁵. Few commonalities exist among the biosynthetic pathways that generate these plant defence compounds other than the fact that they share jasmonate or salicylate as initiating signals. The ability of a generalist to respond

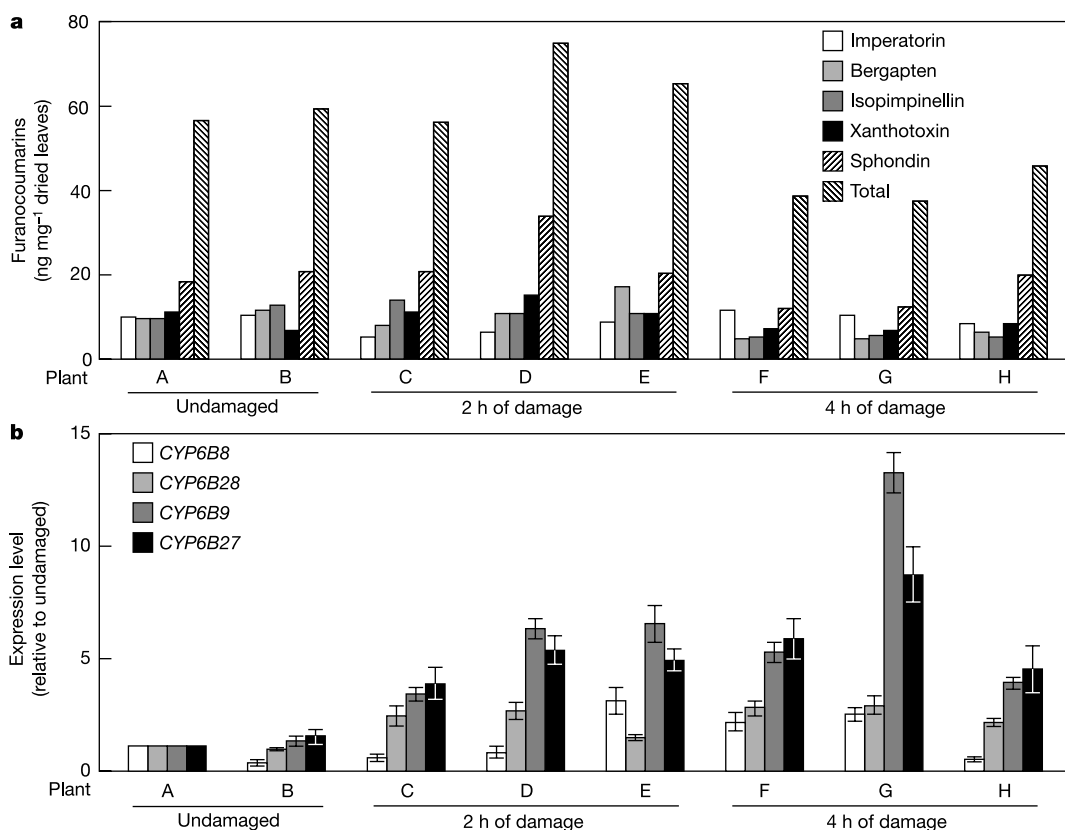


Figure 3 *CYP6B* activation by feeding on damaged celery leaves. **a**, Content of total and individual furanocoumarins for each plant. **b**, Relative induction and standard deviations for three independent RT-PCR amplifications. Total RNAs from fifth instars fed for 48 h on

leaves that were previously undamaged or damaged for 2 or 4 h by starved fourth instars were amplified by RT-PCR and analysed as described in Methods.

to these signals by upregulating several detoxification genes may maximize its ability to counter its host's response to damage, irrespective of taxon. □

Methods

Test insects

An insecticide-susceptible laboratory strain of *H. zea*, provided by B. R. Barrido (Abbott Laboratories), was used in all studies. We kept insects in an insectary maintained at 28 °C in a 16:8 h light:dark cycle on a semisynthetic control diet containing wheatgerm²⁶.

Signal chemical induction treatment

Artificial diets containing 2.9 or 290 µg g⁻¹ jasmonate (Sigma), 12 µg g⁻¹ or 1.2 mg g⁻¹ salicylate (99%, Aldrich), 1.2 mg g⁻¹ methylparaben (Sigma), or 1.2 mg g⁻¹ *p*-hydroxybenzoic acid (Sigma) were provided to 30 newly moulted fifth instars. The low concentrations of jasmonate and salicylate were selected on the basis of endogenous amounts of jasmonate and salicylate found in the host plants of *H. zea*^{4,21} and the high concentrations were selected to maximize the likelihood of detecting an upper limit on the response. After 48 h, midguts and fatbodies were dissected out and total RNAs were isolated from each type of tissue using guanidine-HCl extraction²⁷ and then resuspended in diethyl pyrocarbonate (DEPC)-treated water.

Celery damage and induction treatment

We grew nine celery plants individually in pots under laboratory conditions for 3 weeks to ensure that they were free of herbivore and pathogen infestation. Eight of them were free of infestation and were assigned randomly to one of three groups: undamaged control, 2 h of damage and 4 h of damage. For each plant, four stems with fully expanded pairs of leaflets and a terminal leaflet were chosen for treatment. On each stem, two fourth instars that had been starved for 4 h were confined to the second pair of leaflets by two small clip cages, with one larva per leaflet. For the undamaged controls, clip cages without larvae were placed on the second pair of leaflets on each stem for 4 h. After damage treatments, the second pair of leaflets was removed from each treated stem. We used one damaged leaflet to feed a newly moulted fifth instar that had been starved for 4 h. We pooled another leaflet with the other three leaflets from the same plant, oven-dried them at 50 °C for 24 h and used them for furanocoumarin determination. After 48 h of feeding on the damaged leaves, larvae were killed and the midguts and fatbodies were removed. The midguts from the four larvae fed the damaged leaves from the same plant were pooled together and total RNA was isolated as described²⁷.

RNA and furanocoumarin analysis

We carried out RNA isolation and RT-PCR gel blot analyses as described¹⁷. For each RNA sample, three independent RT-PCR amplifications were carried out. For furanocoumarin assay, all leaf samples were weighed separately and ground to a fine powder with a plastic rod inside 1.5-ml Eppendorf tubes. Furanocoumarins were extracted, separated and quantified as described²⁸.

Jasmonate and salicylate protection bioassay

Newly moulted fourth instars (270) from the University of Illinois laboratory colony were divided randomly into three groups (90 larvae per group) and reared individually in plastic cups with fresh control diets or supplemented diets containing 2.9 µg g⁻¹ jasmonate or 12 µg g⁻¹ salicylate. After 12 h of exposure to plant signal molecules, each group was divided further into three subgroups (30 larvae per subgroup, three replicates of 10 insects) that were transferred to plastic cups with fresh control diets, diets containing 0.5% xanthotoxin, or celery leaves. Initial weights were recorded for every individual. All larvae were weighed again on the third day after transfer to experimental diets to determine weight gain. We monitored survival and developmental stage daily until all larvae had either pupated or died. Differences in weight gain, duration of fourth and fifth instars, mortality, pupal weight and percentage of pupation among the treatments were evaluated by ANOVA, followed by modified least significant difference test (LSD *t*-test), with the significance level set at *P* < 0.05 using the SAS statistics program.

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A biological role for prokaryotic ClC chloride channels

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An unexpected finding emerging from large-scale genome analyses is that prokaryotes express ion channels belonging to molecular families long studied in neurons. Bacteria and archaea are now known to carry genes for potassium channels of the voltage-gated, inward rectifier and calcium-activated classes^{1–3}, ClC-type chloride channels⁴, an ionotropic glutamate receptor⁵ and a sodium channel⁶. For two potassium channels and a chloride channel, these homologues have provided a means to direct structure determination^{3,7–9}. And yet the purposes of these

ion channels in bacteria are unknown. Strong conservation of functionally important sequences from bacteria to vertebrates, and of structure itself¹⁰, suggests that prokaryotes use ion channels in roles more adaptive than providing high-quality protein to structural biologists. Here we show that *Escherichia coli* uses chloride channels of the widespread ClC family in the extreme acid resistance response. We propose that the channels function as an electrical shunt for an outwardly directed virtual proton pump that is linked to amino acid decarboxylation.

The extreme acid resistance (XAR) response allows enteric bacteria to survive for about 1 h in the strongly acidic environment (pH 2–3) of the stomach, a condition essential for gut colonization by benign microorganisms and for virulence of pathogenic ones^{11–14}. In *E. coli* one of the best-characterized XAR mechanisms, the glutamate system, is based on two key genes that work together as a virtual proton pump: *gadB*, a glutamate α -decarboxylase; and *gadC*, a putative amino acid antiporter^{15–17}. In this mechanism, when a stationary-phase bacterium encounters pH 2–3, extracellular glutamate enters the cell through the antiporter, where it is decarboxylated to form GABA (γ -amino butyric acid) in a reaction that consumes a proton. GABA is extruded from the cell through the antiporter and is accompanied passively by membrane-permeant CO₂. In this way, the persistent proton influx that arises from high extracellular acidity is continually counteracted in the cytoplasm by decarboxylation-linked proton utilization. *E. coli* also achieves XAR through an alternative, analogous stationary-phase arginine system linked to the decarboxylation of arginine through the *adiA* decarboxylase to form agmatine (Agm)^{11,13}; although mandated by physiological necessity, the molecular identity of the arginine/agmatine antiporter is currently unknown.

The *E. coli* genome has at least two genes, *eriC* and *mriT* (unknown function designators, yadQ/b0155 and ynfj/b1592, respectively) that encode homologues of the ubiquitous ClC family

of Cl[−] channels^{4,8,18}. *E. coli* strains in which either one of these genes has been deleted show no notable phenotype, but a double-knock-out strain ($\Delta 2$) is severely compromised in its ability to withstand an extreme acid challenge that grossly mimics the bacterium's gastric experience (HCl, pH 2.5, 2 h, 37 °C, minimal medium; Fig. 1). Stationary-phase wild-type bacteria are killed by strong acid in the absence of either glutamate or arginine (<0.1% survival), but supplementation of the acid-challenge medium with either of these amino acids leads to robust survival (30–90%; ref. 15 and Fig. 1). In the $\Delta 2$ strain, however, glutamate or arginine elicits only modest survival (<3%). Neither of the single ClC deletion mutants is impaired in XAR, and delivery of *eriC* on a low-copy plasmid *in trans* rescues XAR in the $\Delta 2$ strain, with rescue being more efficient for the arginine system than for the glutamate system. Thus, the two *E. coli* ClC channels act redundantly. In the experiments described below, therefore, we have focused exclusively on *eriC* because the protein product of this gene, unlike that of *mriT*, is readily over-expressed and manipulated^{4,9}.

Why do cells lacking ClC channels fail to survive extreme acid shock? Both glutamate and arginine decarboxylases, assayed in soluble extracts, are fully competent in the $\Delta 2$ strain (data not shown), but the coupled membrane-transport rates of the amino acids and their decarboxylation products are substantially lower than in wild type. At pH 2.5 in the presence of extracellular glutamate (Fig. 2a) or arginine (Fig. 2b), wild-type *E. coli* release GABA or agmatine into the medium, concomitant with the stoichiometric disappearance of the corresponding decarboxylation substrate; no such transport occurs at neutral pH. In the $\Delta 2$ strain, initial rates of transport are 50 and 93% lower than in wild type for the glutamate and arginine systems, respectively; expression of *eriC* in the $\Delta 2$ strain restores these transport rates fully for the arginine and partially for the glutamate system (Fig. 2c, d). This lower level of rescue of GABA transport might possibly explain the partial rescue

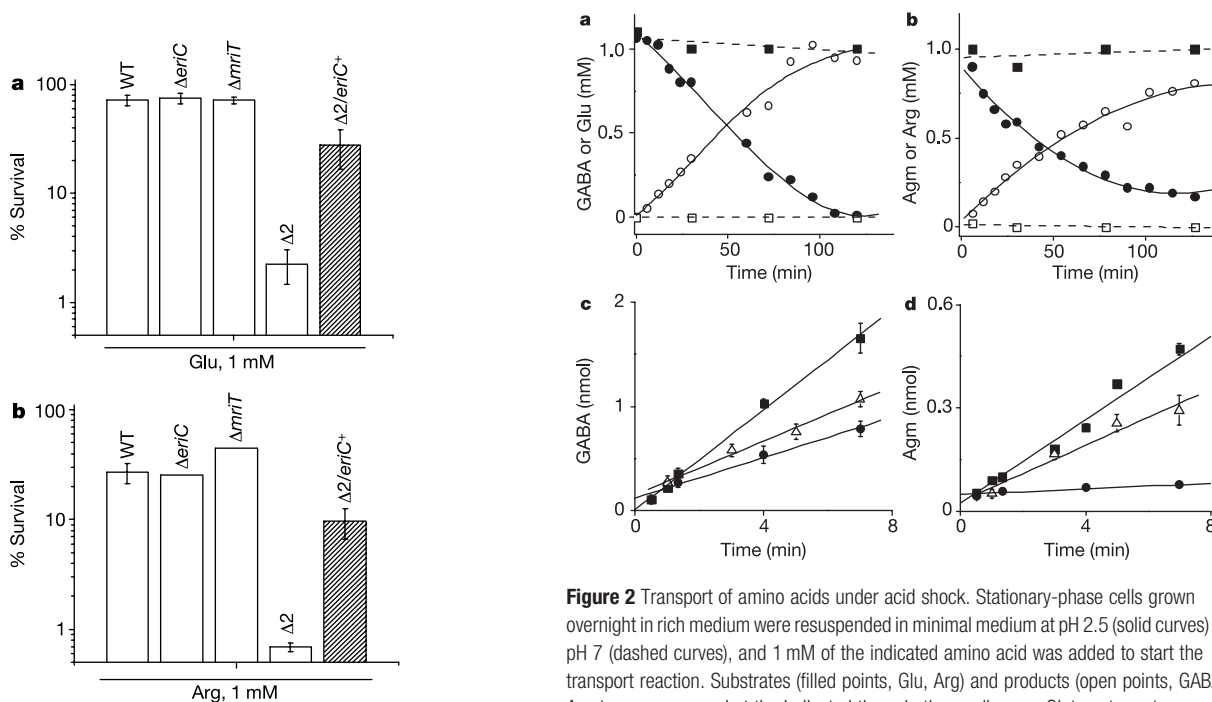


Figure 1 Survival after extreme acid challenge. Percentage of cells surviving extreme acid shock is shown for various *E. coli* strains, in the presence of either 1 mM glutamate (a) or 1 mM arginine (b). Hatched bars denote $\Delta 2$ strain into which *eriC* had been introduced *in trans*. Each bar represents mean $\pm$ s.e.m. of 3–4 independent experiments. Survival in the absence of either amino acid was less than 0.1%. WT, wild type.

Figure 2 Transport of amino acids under acid shock. Stationary-phase cells grown overnight in rich medium were resuspended in minimal medium at pH 2.5 (solid curves) or pH 7 (dashed curves), and 1 mM of the indicated amino acid was added to start the transport reaction. Substrates (filled points, Glu, Arg) and products (open points, GABA, Agm) were measured at the indicated times in the medium. a, Glutamate system (1.5×10^8 cells per ml). b, Arginine system (5×10^8 cells per ml). c, d, Initial rates of product release were measured for GABA (c) and agmatine (d), with 2.3×10^8 cells per ml. Filled squares, wild type; filled circles, $\Delta 2$ strain; open triangles, $\Delta 2$ strain with *eriC* introduced on rescue plasmid. Initial transport rates of the wild type represent roughly 10^4 and 3×10^3 molecules per s per cell for glutamate and arginine systems, respectively. Error bars represent the s.e.m. of 3–4 experiments.

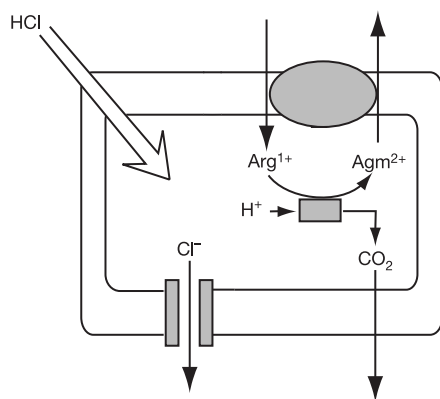


Figure 3 Model of Cl^- channel involvement in XAR. The three essential molecular components of XAR in *E. coli* (grey-shaded shapes) are highlighted: an amino acid decarboxylase (rectangle); a coupled, electrogenic amino acid antiporter (oval); and a Cl^- channel (vertical rectangles). The low-pH challenge that leads to HCl influx (through unknown pathways) is indicated by the wide arrow. The model is for the arginine/arginine system, with the probable charge forms of these amino acids; an identical situation would apply to glutamate/GABA, with the charge forms described in the text.

of glutamate-dependent survival by *eriC*. Thus, the deletion-induced deficit in XAR is traceable to a defect in the ability of the antiporters to deliver substrates to and remove products from the proton-consuming decarboxylases.

Why does transport of these amino acids require Cl^- channels? We propose the obvious mechanism: that the countertransport processes are electrogenic and effectively move positive charge outward (for example, Arg^+ exchanging for Agm^{2+} , Glu^- for GABA^0 , or Glu^0 for GABA^+); therefore, an electrical shunt is needed to prevent the excessive inner-membrane hyperpolarization that would otherwise paralyse the system (Fig. 3). A Cl^- channel fulfilling this role would make physiological sense in the context of the HCl-rich stomach, where Cl^- ions can leak into the cell along with protons and where membrane-permeant molecular HCl may exist at a concentration high enough to account for substantial electroneutral influx of Cl^- ¹⁹. At present, we have no direct evidence that Cl^- is the physiological anion here; however, ClC channels in general²⁰ and *eriC* in particular²¹ are impermeant to the only abundant alternative monoanion, H_2PO_4^- , in the system. An essential feature of this mechanism is that the α -carboxyl group of each amino acid must be deprotonated to bind to and be transported by the antiporter, otherwise the coupled influx, decarboxylation and efflux would fail to consume a cytoplasmic proton. It is this requirement that forces the system to be electrogenic. The high basal glutamate/GABA transport observed in the $\Delta 2$ strain (Fig. 2c) may represent an influx of glutamate with a protonated α -carboxyl group—the main form of the substrate at pH 2.5; such ‘slippage’ would be electro- and proton neutral and would neither contribute to nor detract from survival in acid.

This mechanism indicates that the Cl^- channels should themselves be tightly regulated. Under normal growth conditions near neutrality, a high inner-membrane electrical conductance would be disruptive to energy metabolism, but the Cl^- channels must be called promptly into action by acid shock. We therefore examined whether the channels are activated directly by strong acid. We reconstituted purified *eriC* protein into liposomes and followed electrogenic Cl^- influx as a function of pH (ref. 4). Around neutrality, the channel mediated a low but nonzero uptake of Cl^- ; this rate increased about tenfold as the pH was lowered below pH 5 (Fig. 4). These experiments were carried out under symmetrical pH conditions, so they do not attempt to mimic the

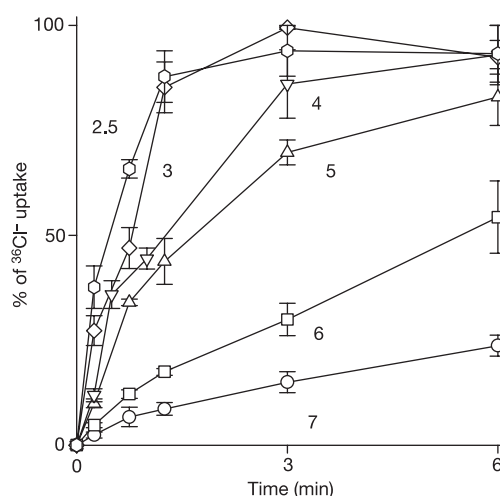


Figure 4 Activation of *eriC* channels at low pH. Time course of $^{36}\text{Cl}^-$ influx in *eriC*-containing liposomes was followed at the pH values indicated. Uptake is normalized to the steady-state uptake observed at 10–60 min (equivalent to 3–8% of total counts, except for the lowest pH uptakes, which were roughly fivefold lower in counts). Control liposomes lacking *eriC* show no significant uptake⁴.

(unknown) pH gradient across the inner membrane of the acid-shocked bacterium, nor do they distinguish *eriC* channels on the basis of their (unknown) orientation in the liposome membrane; however, they do demonstrate that these Cl^- channels are activated under acid-shock conditions, as is predicted for this Cl^- channel to operate coherently towards its biological purpose.

The electrical shunting function of these bacterial ClC channels is reminiscent of roles proposed for several mammalian ClC homologues in distinctly eukaryotic contexts, such as catalysing the counterion currents that accompany primary proton fluxes in endosomal and lysosomal acidification^{22–24}, as well as in neurotransmitter packaging in central neurons²⁵. It is also worth noting that the *E. coli* strain O157:H7 almost certainly uses an XAR mechanism identical to that described here²⁶, and similar mechanisms may operate in other pathogenic enteric bacteria including species of *Shigella*, *Vibrio*, *Yersinia* or *Salmonella*, all of which possess genes for ClC channels. If these microorganisms are also found to use this strategy for XAR, then prokaryotic ClC channels in such pathogens may be viewed as *bona fide* virulence factors. □

Methods

Gene manipulations

Gene deletions were made in *E. coli* wild-type strain MG1655 using the one-step gene inactivation method²⁷, and standard P1 transduction²⁸ was used to transfer genotypes between strains. We confirmed the deletions by sequencing. Single-gene rescue experiments were done in low-copy plasmid pWSK29 (ref. 29), with the *eriC* gene under the control of a *lac* promoter; in most experiments, expression of *eriC* was induced by 5 mM isopropylthiogalactoside before the acid challenge.

Acid shock

We tested glutamate and arginine systems essentially as described¹². Cultures of wild-type or $\Delta 2$ cells were grown for 17–20 h to stationary phase in BHI or Luria broth medium, each supplemented with 20 mM glucose. A 10- μl aliquot of this culture was diluted into 1 ml of acid-challenge medium (40 mM KCl, 80 mM KH_2PO_4 , 33 mM H_3PO_4 , 1.7 mM sodium citrate, 20 mM glucose, pH 2.5) and incubated at 37 °C for 2 h. We spread the cells on LB plates and counted colonies after overnight growth.

Transport assays

Stationary-phase cells were collected by centrifugation and resuspended in pre-warmed acid-shock medium to a final cell density of between 10^8 and 3×10^8 cells per ml. Transport was initiated by adding 1 mM glutamic acid or arginine. Samples (110 μl) were removed at desired intervals and neutralized by rapidly adding 11 μl of 1.1 M KOH, which instantly arrests amino acid transport. The cells were collected by centrifugation, and the supernatants were assayed for amino acids by high-performance liquid chromatography,

using a C₁₈ column and detection at 254 nm, after pre-column derivitization with phenylisothiocyanate³⁰.

³⁶Cl[−] fluxes in eriC-reconstituted liposomes

We carried out overexpression, purification and reconstitution of eriC as described⁴. Influx of ³⁶Cl[−] into liposomes comprising *E. coli* polar lipids (Avanti, 25 mg ml^{−1}) and eriC (1.5 µg mg^{−1} lipid) was carried out under concentrative uptake conditions⁴, except that solutions were buffered with 25 mM citrate/phosphate and were adjusted to the desired pH with NaOH.

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Competence to replicate in the unfertilized egg is conferred by Cdc6 during meiotic maturation

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Meiotic maturation, the final step of oogenesis, is a crucial stage of development in which an immature oocyte becomes a fertilizable egg¹. In *Xenopus*, the ability to replicate DNA is acquired during maturation at breakdown of the nuclear envelope² by translation of a DNA synthesis inducer that is not present in the oocyte^{2,3}. Here we identify Cdc6, which is essential for recruiting the minichromosome maintenance (MCM) helicase to the pre-replication complex, as this inducer of DNA synthesis. We show that maternal *cdc6* mRNA but not protein is stored in the oocyte. Cdc6 protein is synthesized during maturation, but this process can be blocked by degrading the maternal *cdc6* mRNA by oligonucleotide antisense injections or by translation inhibition. Rescue experiments using recombinant Cdc6 protein show that Cdc6 is the only missing replication factor whose translation is necessary and sufficient to confer DNA replication competence to the egg before fertilization. The licence to replicate is given by Cdc6 at the end of meiosis I, but the cytostatic factor (CSF) pathway, which maintains large amounts of active Cdc2/Cyclin B2, prevents the entry into S phase until fertilization.

In most species, oocytes arrest at the prophase stage of the first meiotic division and are not fertilizable. Hormone stimuli induce the resumption of meiosis, with completion of meiosis I and arrest in meiosis II at the metaphase stage in vertebrates. At this stage, the mature oocyte can be fertilized and embryonic development initiated. Analysis of replication initiation factors present in the oocyte showed that Cdc6, an essential factor involved in the assembly of the MCM helicase complex at DNA replication origins^{4,5}, was undetectable in fully grown oocytes but present in mature oocytes and activated eggs (Fig. 1a). By contrast, other initiation proteins analysed were present in the oocyte (Fig. 1b), either nearly exclusively in the nucleus (MCM subunits, RPA70 and RPA34), or also in the cytoplasm (Geminin, Cdt1, Cdc7, ORC and Cdc45; see Supplementary Information Fig. 1). The electrophoretic migration of Cdc6 shifts downwards in interphasic egg extracts (Fig. 1a), in agreement with previous observations^{6–8}.

Although Cdc6 was absent in the oocyte, we detected a relatively large amount of the protein, about 5 ng, in each unfertilized egg, which is comparable to the value of 3 ng reported previously⁶. As Cdc6 is an essential factor for replication licensing, we next determined at what point in maturation it was accumulated. In maturation induced by progesterone (Fig. 1c, d), Cdc6 protein became detectable soon after germinal vesicle breakdown (GVBD; 3 h after progesterone addition), when MCM4 is phosphorylated by maturation-promoting factor (MPF) or cdc2/cyclin B^{9,10}. Inhibition of protein synthesis by cycloheximide added before GVBD, but not 1 h afterwards, resulted in the absence of Cdc6 in the egg (Fig. 1d). These data show that Cdc6 protein is missing in the oocyte (Fig. 1b) and starts to accumulate during maturation.

Although Cdc6 protein was absent in the oocyte, northern blots showed that *cdc6* mRNA was stored as a maternal mRNA during oogenesis (Fig. 2a). In addition, we detected a shift in electrophoretic mobility of the *cdc6* mRNA soon after GVBD (Fig. 2a), consistent with a polyadenylation process occurring during maturation. This shift was still detected if cycloheximide was added 1 h after GVBD, but was extremely reduced if cycloheximide was added

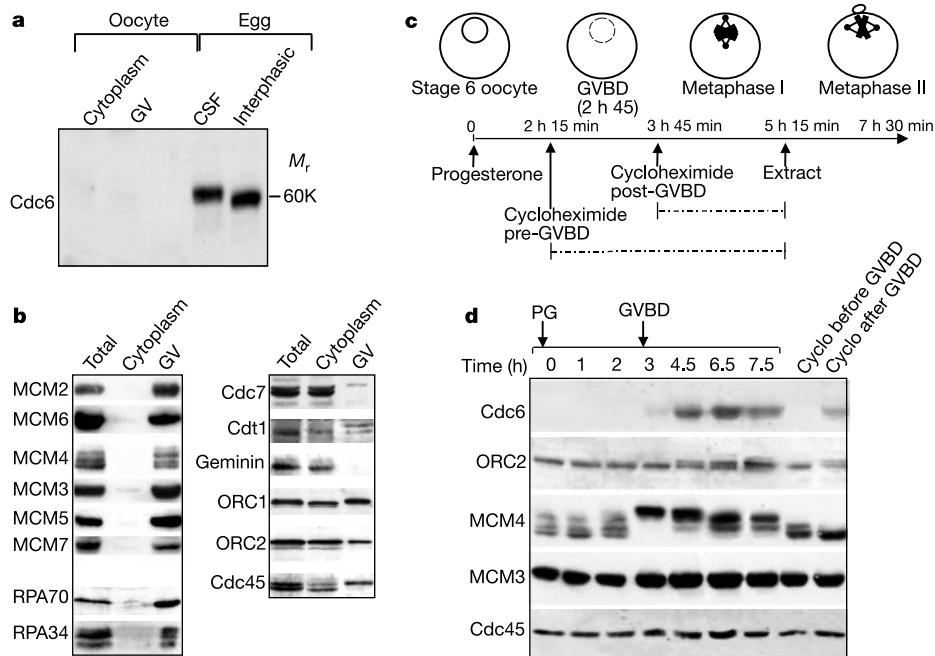


Figure 1 Cdc6 is missing in *Xenopus* oocytes but accumulates during oocyte maturation soon after GVBD. Total oocyte, cytoplasmic and germinal vesicle (GV) fractions, low-speed CSF and interphasic extracts were prepared and analysed by SDS-PAGE. Immunoblots were revealed with antibodies directed against Cdc6 (**a**) and other proteins of the pre-replication complex (**b**). **c**, Stage VI oocytes were induced to mature by adding

progesterone (PG). Some oocytes were treated with cycloheximide either 30 min before GVBD or 1 h after GVBD, and collected at the metaphase I/metaphase II transition (2.5 h after GVBD). **d**, Extracts from five oocytes were separated by SDS-PAGE and examined by immunoblotting.

30 min before GVBD, consistent with the respective absence and presence of Cdc6 protein in maturing oocytes (Fig. 1d). Actinomycin D present during maturation did not inhibit this shift (Fig. 2a), suggesting that synthesis of Cdc6 is regulated at the translational level by polyadenylation of a maternal *cdc6* mRNA pool. In addition, the accumulation of Cdc6 protein in the egg was blocked by injecting antisense *cdc6* oligonucleotides into the oocyte before maturation, but not by injecting the corresponding sense oligonucleotides (Fig. 2b). These results suggest that synthesis of Cdc6 protein during oocyte maturation relies on translational control of *cdc6* mRNA stored in the oocyte.

To determine whether Cdc6 was the missing replication factor sufficient to confer the competence for DNA replication to the egg, we examined whether recombinant Cdc6 protein was sufficient to

provide this competence. We purified recombinant Cdc6 expressed from baculovirus-infected cells and showed that it was active in rescuing DNA replication in *Xenopus* egg extracts that were depleted of Cdc6 through a purified polyclonal antibody specific to Cdc6 (Methods and Supplementary Information Fig. 2). We used this purified recombinant protein in the *in vivo* rescue experiments described below.

The activity of the missing DNA synthesis inducer that is synthesized during maturation is normally repressed by MPF to prevent DNA replication between meiosis I and meiosis II (ref. 3). Addition of cycloheximide 1 h after GVBD prevented the reaccumulation of MPF and consequently induced unscheduled DNA replication between meiosis I and meiosis II (ref. 3). To address whether this induced ability to replicate required the synthesis of

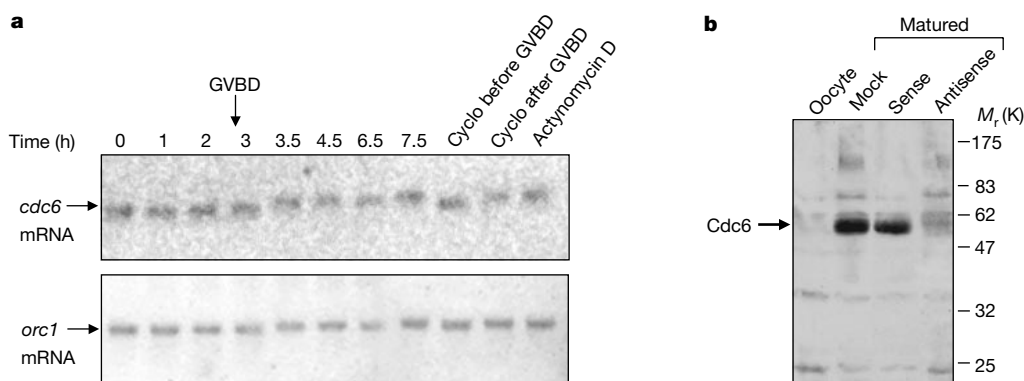


Figure 2 *cdc6* mRNA stored in the oocyte is translated after GVBD. **a**, Oocytes were induced to mature by adding progesterone (PG). Some oocytes were also treated with cycloheximide as described in Fig. 1. RNA was analysed by northern blot. **b**, Stage VI oocytes were injected with 50 ng of a 25-nucleotide sense or the corresponding antisense

cdc6 oligonucleotide, or water as a control (mock), and induced to mature by progesterone. Matured oocytes were collected 12 h after progesterone stimulation and analysed for Cdc6 protein.

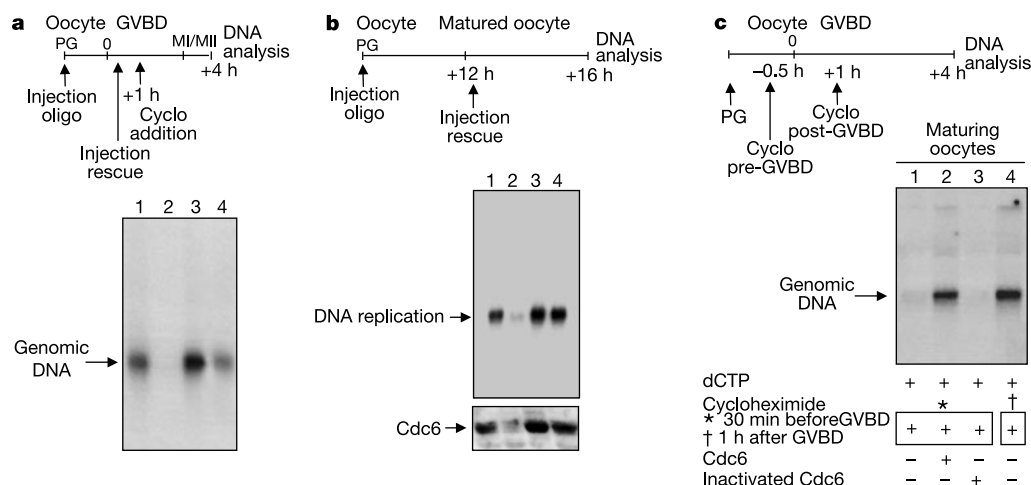


Figure 3 Cdc6 is the DNA replication induced activity acquired *in vivo* during maturation. **a**, Oocytes injected with 25 nl of a 2 mg ml⁻¹ solution of purified *cdc6* sense oligonucleotide (lane 1) or the corresponding antisense oligonucleotide (lanes 2 and 3), and control oocytes injected with water (lane 4) were induced to mature. Rescue experiments were assayed by a second injection soon after GVBD that contained 0.25 µCi of [α -³²P]dCTP (all lanes) and either boiled inactivated Cdc6 (lane 2) or intact purified Cdc6 (lane 3). Maturing oocytes were incubated in cycloheximide added 1 h after GVBD to test for ability to induce DNA replication between meiosis I and meiosis II. DNA synthesis was analysed 4 h after GVBD as described⁵ by agarose gel electrophoresis. **b**, Oocytes injected with water (lane 1), antisense oligonucleotides (lanes 2 and 3) or sense

oligonucleotides (lane 4) were allowed to mature fully for 12 h. DNA replication was analysed by injecting 0.25 µCi of [α -³²P]dCTP in the absence (lanes 1, 2 and 4) or presence (lane 3) of recombinant Cdc6 protein. DNA synthesis was analysed 4 h after injection by agarose gel electrophoresis. Injected oocytes were also analysed by immunoblot with Cdc6 antibody (bottom). **c**, Oocytes induced to mature were treated with 250 µg ml⁻¹ cycloheximide added 30 min before GVBD (+) or 1 h after GVBD (†). Maturing oocytes were injected soon after GVBD with 0.25 µCi of [α -³²P]dCTP without (lanes 1 and 4) or with active Cdc6 (lane 2) or inactivated Cdc6 (lane 3). DNA synthesis was analysed 4 h after GVBD by agarose gel electrophoresis.

Cdc6 during maturation, we first determined whether *cdc6* mRNA depletion by antisense oligonucleotide could prevent this unscheduled replication. Addition of cycloheximide 1 h after GVBD induced DNA replication between meiosis I and meiosis II, as previously reported³, but this induction was inhibited by *cdc6* antisense oligonucleotides (Fig. 3a). In addition, injecting recombinant Cdc6 rescued the induction of unscheduled replication (Fig. 3a).

We next determined whether Cdc6 was sufficient to confer competence to replicate *in vivo* to the matured oocyte. After maturation *in vivo*, parthenogenetic activation triggered by micropipette injection induces DNA replication (refs 2, 11, 12, and Fig. 3b, lane 1). Injecting *cdc6* antisense oligonucleotides into the oocyte before maturation inhibited DNA replication (Fig. 3b, lane 2), but microinjection of Cdc6 protein after maturation was sufficient to rescue DNA replication (Fig. 3b, lane 3) with an efficiency similar to control oocytes treated with sense oligonucleotides (Fig. 3b, lane 4). We conclude that Cdc6 is the missing factor synthesized during oocyte maturation, sufficient to confer both replication-induced ability between meiosis I and meiosis II and competence to replicate in the unfertilized egg.

Previous work has shown that maturing oocytes can replicate DNA after meiosis I by cycloheximide treatment 60 min after GVBD but not 30 min before GVBD³. We reproduced these data, confirming that competence for DNA replication *in vivo* is acquired shortly after GVBD and is dependent on translation (Fig. 3c, lanes 1 and 4). We also showed that microinjection of Cdc6 after translation inhibition 30 min before GVBD is sufficient to rescue induced-replication ability between meiosis I and meiosis II (Fig. 3c, lanes 1–3).

These *in vivo* observations could be reproduced *in vitro*, in oocyte extracts prepared between meiosis I and meiosis II (refs 13, 14 and Methods). Oocytes were matured *in vitro*, and cycloheximide was added 30 min before or 60 min after GVBD (Fig. 4a). Extracts were made 2.5 h after GVBD and sperm nuclei were added to follow DNA replication ability. If cycloheximide was added 60 min after GVBD, then DNA replication occurred (Fig. 4b, c), as expected, in a reaction that was totally sensitive to aphidicolin (Fig. 4c). If

cycloheximide was added 30 min before GVBD, then Cdc6 was not translated (Fig. 1d) and extracts were incompetent to replicate (Fig. 4b, c). But the addition of recombinant Cdc6 to the extract was sufficient to rescue replication in an aphidicolin-sensitive reaction (Fig. 4c). Furthermore, the simple addition of recombinant Cdc6 to oocyte extracts made at GVBD was sufficient to confer DNA replication competence with an efficiency close to that obtained in egg extracts (Fig. 4d). Finally, the ability to replicate conferred between GVBD and meiosis I to meiosis II transition is inducible by Ca²⁺, as in the unfertilized egg (Supplementary Information, Fig. 3). These results verify that Cdc6 is the missing DNA replication factor, which starts to be translated at GVBD and is necessary and sufficient to confer to the egg the ability to replicate embryonic DNA after fertilization, when the MPF activity is suppressed. Similar observations were obtained by Coué *et al.*³⁰ with similar conclusions.

We have identified Cdc6 as the DNA synthesis inducer that has been postulated to give competence to replicate during meiotic maturation before fertilization^{2,3}. *cdc6* is expressed as a maternal mRNA in oocytes, and the protein accumulates during progesterone-induced maturation. This regulation occurs at the translational level, similar to the regulation of *c-mos* involved in the CSF activity that maintains the high level of MPF activity necessary for the meiotic arrest of unfertilized eggs^{15–18}. The unmasking of specific maternal mRNAs involved in two crucial opposite stages of the cell cycle, initiation of DNA replication and mitosis, therefore contributes to the final stage of oogenesis that leads to the unfertilized egg.

First, competence to form the replication complex is acquired shortly after GVBD with synthesis of Cdc6 (Fig. 5). Cdc6 absence before this last step of oogenesis is likely to provide a way to prevent origin complex formation during the long period of oocyte growth. This acquisition of a licence to replicate at the end of meiosis I might be considered unexpected because preventing DNA replication at this step is crucial to producing a haploid genome. But a negative control of this acquired licence might be achieved by *c-mos*, which starts to accumulate just before GVBD (Fig. 5). The Mos/mitogen-activated protein kinase pathway prevents DNA replication between meiosis I and meiosis II, by prematurely reactivating Cdc2/Cyclin B

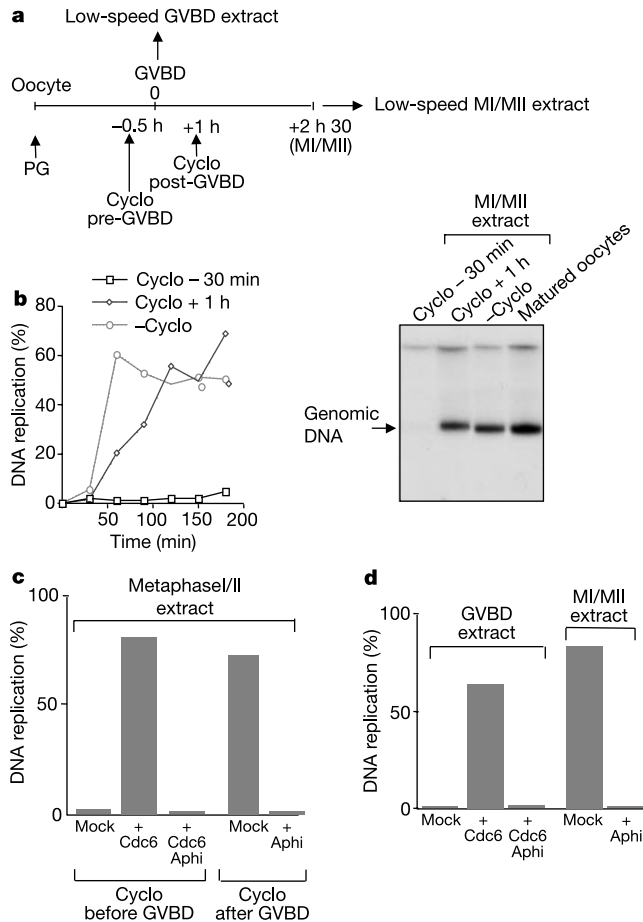


Figure 4 Cdc6 protein is the missing factor acquired soon after GVBD that confers to the egg the competence to replicate. **a**, Stage VI oocytes induced to mature were either left untreated (—cyclo) or treated with 250 $\mu\text{g ml}^{-1}$ cycloheximide 30 min before GVBD (cyclo, —30 min) or 1 h after GVBD (cyclo +1 h). Oocytes were collected either at the GVBD stage, or at the metaphase I/metaphase II (MI/II) transition (2.5 h after GVBD). Matured oocytes were also collected 12 h after PG addition, and low-speed extracts were prepared. **b**, Sperm nuclei were added to the extracts and DNA synthesis was followed over time and by agarose gel electrophoresis after 2 h. **c**, DNA replication assay was carried out in MI/II extracts as in **b**. Rescue experiments were carried out by adding purified baculovirus recombinant Cdc6. Aphidicolin (aphi, 100 $\mu\text{g ml}^{-1}$) inhibited DNA replication at the same concentration as in *Xenopus* egg extracts. **d**, DNA replication assays were carried out in GVBD extracts and Cdc6 rescue experiments were carried out as in **c**.

after metaphase^{15–20}, thereby allowing meiotic reduction in chromosome number. Consequently, *c-mos* activity during maturation can be considered as a second, superimposed regulation to prevent DNA replication origins firing before fertilization. Geminin, a negative regulator of Cdt1, is present in oocytes as well as in meiosis I/meiosis II extracts from oocytes treated with cycloheximide 1 h after GVBD or from fertilized egg, which are both competent for DNA replication (unpublished data); therefore, its presence cannot explain the repression of DNA replication at these two stages. Emil, a component of the CSF pathway²¹, might be also required in metaphase II arrest. Such coupled regulation, in which the competence to replicate that is given during maturation is repressed until fertilization, represents a way to induce precisely and rapidly the activation of DNA replication immediately after fertilization^{22,23}.

Our data also emphasize that regulating the recruitment of the MCM helicase to origins is an essential rate-limiting step in initiating DNA replication, and strengthen the role of Cdc6 in determining the replication competence in cells after long periods of

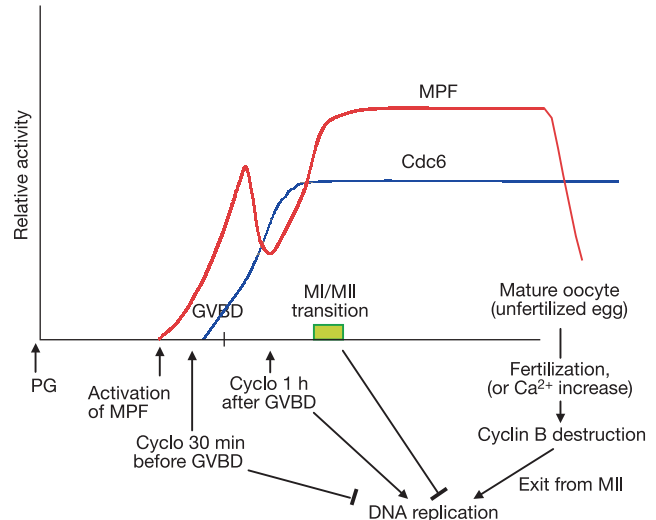


Figure 5 Interplay between replication induced by *cdc6* and its negative control by MPF. Cdc6 is missing in the oocyte but starts to be translated from oocyte *cdc6* maternal mRNA at GVBD during maturation. The second rise of MPF activity prevents activation of DNA replication by Cdc6 during the transition between metaphase I and metaphase II (MI/II). At fertilization, the degradation of Cyclin B2 and the consequent inactivation of MPF permits the onset of DNA replication in the embryo. Cycloheximide added 30 min before GVBD inhibits DNA replication because it totally prevents the accumulation of Cdc6. Cycloheximide added 1 h after GVBD activates DNA replication because it prevents the second rise of MPF activity when Cdc6 has already started to accumulate.

quiescence^{24,25}. The role of Cdc6 in preparing the egg for fertilization is also emphasized by the observation that Cdc6 can induce ability to bind sperm in immature oocytes²⁶, a property that is also acquired during maturation. The induction of two apparently unrelated events, competence to replication and sperm-binding ability (which are both essential for fertilization), by the same protein is unexpected. It might unveil links between DNA replication controls and developmental controls, which may be essential in multicellular eukaryotes. □

Methods

Fractionation of oocytes

We prepared defolliculated oocytes as described¹⁴. Cytoplasmic and germinal vesicle fractions were obtained after manual enucleation either from intact oocytes or from oocytes fixed by 2% trichloroacetic acid (TCA) to prevent leaks from the nucleus. Protein extracts were obtained by grinding oocytes in 100 mM KCl, 5 mM MgCl_2 , 0.1 mM CaCl_2 , 10 mM potassium HEPES, pH 7.7, 50 mM sucrose, 5 $\mu\text{g ml}^{-1}$ leupeptin, 5 $\mu\text{g ml}^{-1}$ pepstatin, 5 $\mu\text{g ml}^{-1}$ aprotinin, 0.5 mM EGTA and 0.3% Triton X-100, followed by centrifugation for 5 min at 8,000g, and were analysed by SDS-PAGE.

Oocyte extracts

Low-speed extracts of maturing oocytes were carried out either at GVBD or 2.5 h after GVBD between meiosis I and meiosis II. GVBD, as judged by the appearance of a white spot at the animal pole, started at between 2 h 45 min and 3.5 h. Maturing oocytes extracts were prepared as described for egg extracts¹⁴ in Eppendorf tubes. After the excess buffer was removed, the oocytes were centrifuged at 8,000g for 5 min at 4 °C, and then at 12,000g for 2 min at 4 °C. The upper phase was collected and centrifuged again at 12,000g for 2 min at 4 °C. We collected the extracts and supplemented them with energy mix¹⁴.

DNA replication assays

We injected oocytes with 25 nl of 5 mCi ml^{-1} [α -³²P]dCTP and extracted the DNA 4–5 h after GVBD. Samples with equal numbers of total counts were analysed by 0.8% agarose gel electrophoresis. DNA replication assays were also carried out by quantification of [α -³²P]dCTP incorporation in sperm nuclei used at 1,000 nuclei per ml of extract. We carried out analysis by TCA precipitation and migration on 0.8% agarose gels¹⁴. Purified protein was added at 15 ng per μl of extract to rescue DNA replication activity. We prepared interphasic extract and CSF extract as described^{14,27}.

Northern blot analysis

Total oocyte RNA was extracted by the LiCl method²⁸. We resolved 10 μg of total RNA on a 1% formaldehyde agarose gel and transferred them to Amersham Hybond N+ membrane.

Injections

Stage VI oocytes were injected with 25 nl of a 2 mg ml⁻¹ solution of a purified *cdc6* 25-nucleotide sense (5'-TCCCCACCAAGCAGTCTCGCAAAG-3') or antisense (5'-CTTTGCGAGACTGCTTGGGTGGGA-3') oligonucleotide, and induced to mature by addition of progesterone. Control oocytes were injected in parallel with water. For rescue experiments, a 25-nl second injection containing 5 mCi ml⁻¹ dCTP and 300 µg ml⁻¹ purified Cdc6 protein was injected after GVBD or into matured 12-h oocytes. Maturation was followed by appearance of a clear white spot at the animal hemisphere coupled with manual dissection to check for GVBD.

Cdc6 protein and antibodies

Xenopus His₆-Cdc6 protein was purified from insect cells infected with recombinant baculovirus. We monitored its activity by depletion-rescue experiments as follows: 50 µl of low-speed interphase extract was either depleted with a specific polyclonal antibody to Cdc6 or mock-depleted with pre-immune serum immunoglobulin-γ, as described²⁹. We added purified Cdc6 protein to the depleted extract and measured DNA replication (Supplementary Information Fig. 1). The rabbit polyclonal antibody against Cdc6 and antibodies against Cdt1, MCM4, MCM3, RPA34 and Geminin were obtained by four injections of corresponding recombinant proteins. Other antibodies were generous gifts from J. Blow (ORC1), H. Takisawa (Cdc45, MCM2, MCM5, MCM6 and MCM7), J. Maller (Cdc7), Y. Adachi (RPA70) and J. Walter (ORC2). The *Xenopus* Cdc6 baculovirus⁶ was a generous gift from T. Coleman.

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Cdc6 synthesis regulates replication competence in *Xenopus* oocytes

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The early division cycles of an embryo rely on the oocyte's ability to replicate DNA. During meiosis, oocytes temporarily lose this ability. After a single round of pre-meiotic S-phase, oocytes enter meiosis and rapidly arrest at prophase of meiosis I (G2)¹. Upon hormonal stimulation, arrested oocytes resume meiosis, re-establish DNA replication competence in meiosis I shortly after germinal vesicle breakdown (GVBD), but repress replication until fertilization^{2,3}. How oocytes lose and regain replication competence during meiosis are important questions underlying the production of functional gametes. Here we show that the inability of immature *Xenopus* oocytes to replicate is linked to the absence of the Cdc6 protein and the cytoplasmic localization of other initiation proteins. Injection of Cdc6 protein into immature oocytes does not induce DNA replication. However, injection of Cdc6 into oocytes undergoing GVBD is sufficient to induce DNA replication in the absence of protein synthesis. Our results show that GVBD and Cdc6 synthesis are the only events that limit the establishment of the oocyte's replication competence during meiosis.

The focus of our study is the acquisition of replication competence of *Xenopus* oocytes during meiosis, a question that was first examined by Gurdon in 1967¹. Subsequently, the replication defect of immature *Xenopus* oocytes arrested at prophase of meiosis I was linked to the initiation step rather than the elongation step of DNA replication⁴. *Xenopus* immature oocytes contain functional polymerases that can support semi-conservative replication of single-stranded DNA⁵. Initiation of eukaryotic DNA replication requires the assembly onto the chromatin of pre-replication complexes (pre-RCs) containing Orc, Cdc6, Cdt1 and Mcm proteins^{6,7}. The pre-RCs are then activated by the Cdk2/cyclin E and Cdc7/Dbf4 kinases leading to binding of the Cdc45 protein to the pre-RCs and unwinding of the DNA^{8,9}. To determine whether the initiation defect corresponds to a defect in pre-RC assembly or activation, we looked at the expression of pre-RC components and activating kinases in immature (stage VI) *Xenopus* oocytes. Western blot analysis showed that all but one of the initiation factors tested

were expressed in immature oocytes (Fig. 1a, lane 3). The Orc1 and Orc2 subunits, the Cdt1 and Mcm proteins were present in *Xenopus* stage-VI oocytes at levels comparable to those found in *Xenopus* eggs before fertilization (Fig. 1a, lanes 1–3). The only missing pre-RC component was the Cdc6 protein. Other initiation factors involved in the pre-RC activation step, such as the Cdc7 and Cdc45 proteins, were also found in stage-VI oocytes. The absence of the Cdc6 protein in immature *Xenopus* oocytes suggests that these oocytes cannot assemble pre-RCs and therefore cannot initiate DNA replication.

Next we examined the subcellular localization of the initiation factors present in stage-VI *Xenopus* oocytes (Fig. 1a, lanes 4 and 5). Manual separation of the oocyte nucleus (germinal vesicle) and cytoplasm followed by western blot analysis indicated that all the Mcm proteins were localized in the germinal vesicle. The Cdt1 and Cdc45 proteins were also predominantly nuclear, whereas the Orc subunits and the Cdc7 protein kinase were mostly found in the cytoplasm. The other pre-RC activating kinase, Cdk2/cyclin E, was also found in the cytoplasm (data not shown). The nuclear exclu-

sion of two Orc subunits provides further evidence that the inability of immature oocytes to initiate DNA replication is linked to their inability to assemble pre-RCs. The cytoplasmic localization of the Cdc7 and Cdk2/cyclin E kinases also suggests that even if pre-RCs could form on the chromatin they could not be activated by these two kinases.

Phosphorylation of pre-RC components is one mechanism believed to regulate pre-RC assembly and function during the cell cycle⁶. Among the pre-RC components expressed in the immature oocytes, the Mcm4 protein was the only one that appeared to be phosphorylated. The Mcm4 isoform found in the germinal vesicle (GV) displayed an electrophoretic mobility intermediate between the mobility of the hyperphosphorylated isoforms and that of the hypophosphorylated isoforms found in mitotic and interphasic *Xenopus* egg extracts, respectively (Fig. 1a, Mcm4 in lanes 1, 2 and 5). Phosphatase treatment of the GV Mcm4 isoform increased its electrophoretic mobility, confirming that it was phosphorylated (Fig. 1b). Phosphopeptide mapping analysis indicated that the targeted phosphorylation sites were a subset of the Mcm4 sites phosphorylated during mitosis (Fig. 1c). Cdc2/cyclin B phosphorylates these sites *in vitro*¹⁰; we therefore examined the possibility that Cdc2/cyclin B phosphorylated Mcm4 in the GV. In immature oocytes Cdc2/cyclin B and its coactivator Cdc25 are predominantly cytoplasmic. However, they continuously shuttle in and out of the nucleus leading to a transient activation of the Cdc2/cyclin B kinase inside the GV^{11–13}. We incubated immature oocytes in the presence of leptomycin B (an inhibitor of nuclear export) to increase the concentration of active Cdc2/cyclin B inside the GV¹³ (Fig. 1d, e). As a result, the Mcm4 protein in the GV exhibited a slower gel migration indicating an increase in its phosphorylation level

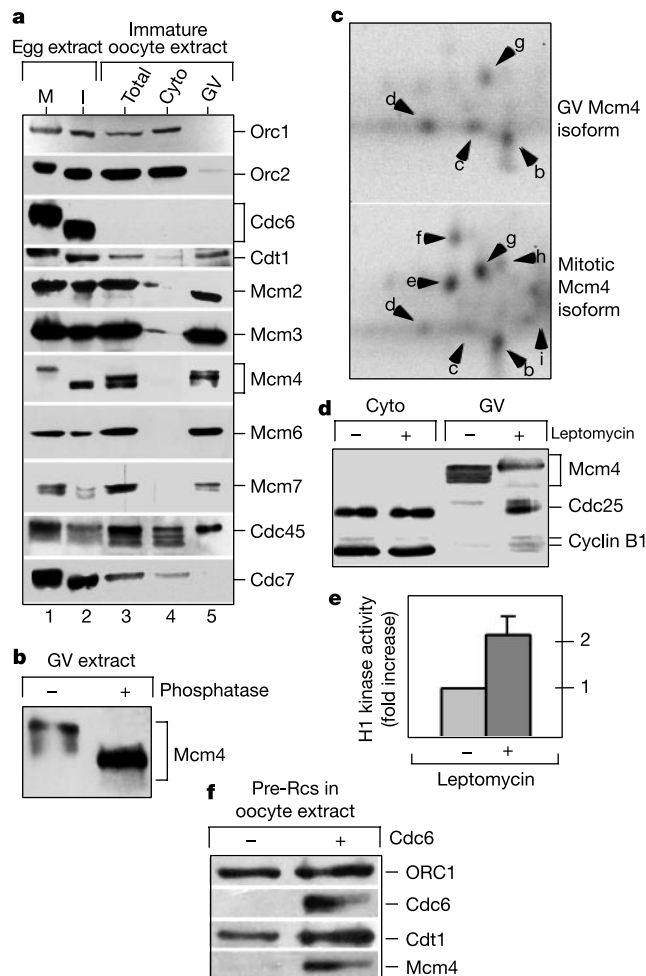


Figure 1 *Xenopus* immature oocytes have a defect in pre-replication complex (RC) assembly. **a**, Western blot of initiation factors in total (lane 3), cytoplasmic (lane 4) or nuclear (lane 5) extracts from immature *Xenopus* oocytes and in mitotic (lane 1) or interphase (lane 2) egg extracts. Cyto, cytoplasm; GV, germinal vesicle. Differences in localization between this work and ref. 28 are addressed in Supplementary Information Fig. 1. **b**, **c**, Phosphatase treatment and tryptic phosphopeptide map of the Mcm4 isoform found in the GV of stage VI oocytes. **d**, Mcm4, Cdc25 and cyclin B1 in cytoplasmic and nuclear extracts of leptomycin-B-treated oocytes. Cyclin B1 migrates as a doublet above a major cross-reactive band²⁹. **e**, H1 kinase activity in the nucleus of immature oocytes treated with or without leptomycin B. **f**, Proteins bound to sperm chromatin incubated in immature oocyte extracts with or without recombinant Cdc6 protein (1.8 ng μl^{-1} extract).

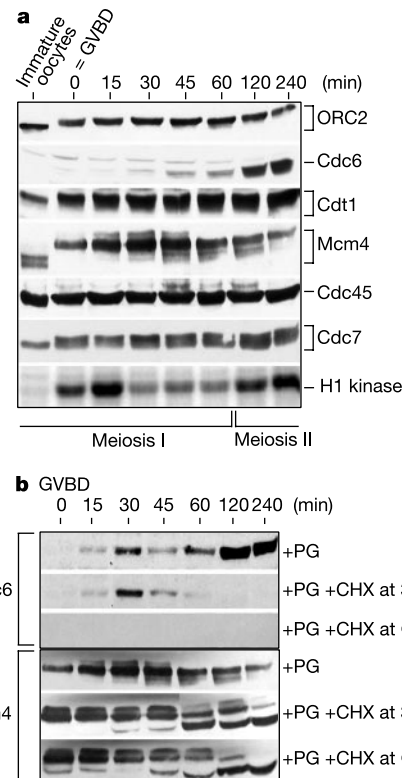


Figure 2 Cdc6 synthesis occurs during meiosis I after germinal vesicle breakdown (GVBD). **a**, Maturing oocytes were collected at different times (shown) after GVBD and subjected to western-blot analysis with the indicated antibodies. Meiosis progression was followed by determining the H1 kinase activity in oocyte extracts. **b**, Progesterone-induced maturation was carried out in the presence or absence of 100 $\mu\text{g ml}^{-1}$ cycloheximide (CHX) added to oocytes either at GVBD or 30 min after GVBD. Mcm4 and Cdc6 expression was analysed by western blotting at the indicated times after GVBD.

(Fig. 1d). Taken together, these results suggest that in immature oocytes the Mcm4 subunit is phosphorylated by Cdc2/cyclin B. Unlike in yeast^{14,15}, phosphorylation of the Mcm4 subunit did not induce nuclear export of the Mcm complex; thus, its role in regulating pre-RC assembly remains to be further characterized.

We describe three ways *Xenopus* oocytes can prevent pre-RC assembly during their prolonged G2 arrest in prophase of meiosis I: (1) absence of Cdc6 protein; (2) compartmentalization of pre-RC components; and (3) phosphorylation of the Mcm4 subunit of the Mcm complex. If these three factors are indeed the only ones preventing pre-RC assembly, we reasoned that pre-RCs should assemble on sperm chromatin incubated in a total immature oocyte extract supplemented with recombinant *Xenopus* Cdc6 protein. In such an extract, nuclear and cytoplasmic initiation factors present in immature oocytes are reunited and the nuclear Mcm4 subunit is dephosphorylated upon contact with cytoplasmic phosphatases. As shown in Fig. 1f, complete pre-RC assembly, as determined by Mcm binding to chromatin, occurred in the presence but not in the absence of added Cdc6 protein. Orc and Cdt1 proteins were the only proteins associated with the chromatin in the absence of Cdc6. We conclude that Cdc6 is the only missing pre-RC component in immature oocytes and that nuclear envelope breakdown is also required for pre-RC assembly. Our results clearly establish that the initiation defect in immature oocytes corresponds to a defect in pre-RC assembly.

Although oocytes normally do not replicate their DNA between meiosis I and meiosis II, they can be artificially forced to do so by the addition of cycloheximide (CHX) 30 to 60 min after GVBD³. Such treatment prevents maturation-promoting factor (MPF) reactivation at the end of meiosis I and therefore progression into meiosis II. Instead, following meiosis I, the oocytes reform nuclei and replicate their DNA. However, if CHX is added at GVBD, oocytes reform nuclei but are unable to replicate their DNA. These different CHX treatments have established that the replication competence of *Xenopus* oocytes arises during meiosis I and requires the synthesis

of one or several essential unknown factors about the time of GVBD³.

To test the possibility that Cdc6 was such an essential factor we first looked at its expression profile during the maturation process without CHX or with CHX added at GVBD, or at 30 min after GVBD. During the normal maturation process induced by progesterone in the absence of CHX, low levels of Cdc6 protein were first detected at GVBD and persisted during meiosis I (Fig. 2a). A significant increase in Cdc6 expression occurred during meiosis II (Fig. 2a) and correlated with the cytoplasmic polyadenylation of the message (data not shown). This difference in the level of Cdc6 expression during maturation was consistently observed with different batches of oocytes. The expression level of the other initiation factors remained constant during maturation but some factors appeared to be phosphorylated at the onset of GVBD and stayed phosphorylated throughout the meiosis II metaphase arrest (see mobility shift of Mcm4, Cdt1, Orc2 and Cdc7 proteins in Fig. 2a).

Addition of CHX to oocytes undergoing GVBD, a treatment that blocks the development of replication competence³, inhibited Cdc6 synthesis but did not affect the expression of other replication factors (Fig. 2b). However, the absence of MPF reactivation after CHX addition led to Mcm4 dephosphorylation (Fig. 2b). Addition of CHX 30 min following GVBD allows the oocytes to replicate their DNA³. Under these conditions, low levels of Cdc6 protein were observed during meiosis I but rapidly disappeared thereafter (Fig. 2b). Again, addition of CHX 30 min after GVBD affected the Mcm4 phosphorylation state but not its expression. The timing of Cdc6 expression during maturation and its changes upon CHX treatment strongly suggest that Cdc6 is an important factor for the development of the oocyte's replication competence.

Next we determined if Cdc6 was the only factor limiting the replication competence of the oocytes after GVBD. We tested the ability of Cdc6 to rescue DNA replication by injecting it into maturing oocytes treated with CHX at GVBD. Under these conditions protein synthesis occurs before GVBD but the synthesis of endogenous Cdc6 protein (Fig. 2b), or any other essential replication factors after GVBD, is prevented. Oocytes undergoing GVBD were treated with CHX and injected with [α -³²P]dCTP and Cdc6 or control proteins. Analysis of chromosomal DNA indicated that oocytes injected with buffer, BSA or recombinant Cdc45 proteins were unable to replicate their DNA after meiosis I (Fig. 3a). On the other hand, injection of the Cdc6 protein induced DNA replication in a concentration-dependent manner (Fig. 3a). Cdc6-induced replication was sensitive to aphidicolin, indicating that DNA replication rather than DNA repair was occurring (Fig. 3b). These findings demonstrate that after GVBD, Cdc6 is the only factor whose synthesis is limiting for the establishment of the oocyte's replication competence.

However, these experiments do not exclude the possibility that before GVBD the synthesis of other factors is also required for the development of replication competence. To address this point, we tested the ability of Cdc6 to induce DNA replication in the absence of protein synthesis. First Cdc6 protein was injected into the cytoplasm or the germinal vesicle of immature oocytes incubated in the presence of CHX. Regardless of the injection site, Cdc6 was unable to induce DNA replication (Fig. 3c). Although this negative result could indicate the required synthesis of other unknown initiation factors, it most probably reflects the inability of immature oocytes to assemble pre-RCs with components that are localized in different cellular compartments. To test this and allow pre-RC assembly in immature oocytes treated with CHX, we induced GVBD by injecting cyclin A protein. Injection of cyclin A activates the pool of pre-MPF (Cdc2/cyclin B) present in the immature oocytes and leads to resumption of meiosis I and nuclear envelope breakdown¹⁶. When we injected a low concentration of cyclin A (50 nM intracellular concentration) in oocytes treated with CHX, the oocytes underwent GVBD but were unable to maintain a high Cdc2 kinase activity and did not proceed toward meiosis II¹⁷ (see

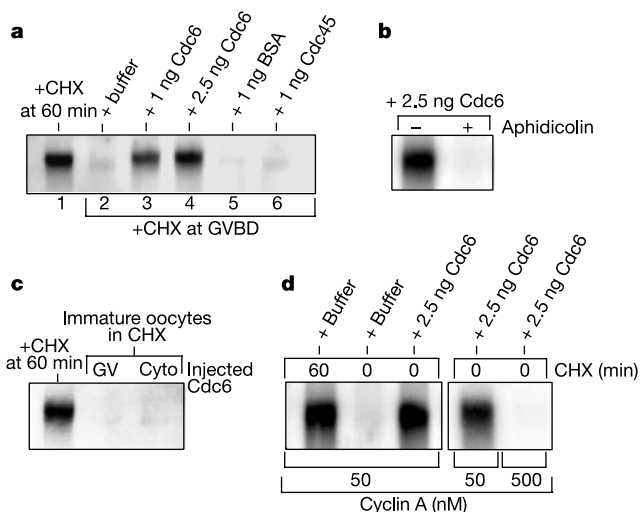


Figure 3 Injection of Cdc6 into maturing oocytes treated with CHX induces DNA replication. **a**, Oocytes undergoing GVBD were injected with a solution containing [α -³²P]dCTP and the indicated recombinant proteins. CHX was added either 1 h after GVBD (lane 1) or at time of GVBD (lanes 2–6). Replicated DNA was analysed 4 h after GVBD. **b**, As in **a**, lane 4, in the presence or absence of 20 μg ml⁻¹ aphidicolin. **c**, Absence of replication in immature oocytes treated with CHX and injected either into the cytoplasm or the germinal vesicle (GV) with [α -³²P]dCTP and Cdc6 protein (2.5 ng). Oocytes treated with progesterone and CHX 60 min after GVBD were used as replication control. **d**, Oocytes were injected with recombinant cyclin A (50 nM or 500 nM intracellular concentration) to induce GVBD. CHX was added either at the time of cyclin A injection (0 min) or 60 min after GVBD. Replication was monitored after the oocytes were injected again at GVBD with [α -³²P]dCTP and 2.5 ng of recombinant Cdc6 protein or buffer.

Supplementary Information Fig. 2). Instead the oocytes entered interphase at the end of meiosis I. Injection of 50 nM of cyclin A alone, in the absence of protein synthesis, did not allow DNA replication during this interphase, but injection of both 50 nM of cyclin A and 2.5 ng of Cdc6 did induce DNA replication (Fig. 3d). However, the ability of Cdc6 to induce DNA replication was inhibited when a tenfold higher concentration of cyclin A was used to induce GVBD (Fig. 3d). This inhibition is most probably the result of the sustained high Cdc2 kinase activity in oocytes injected with 500 nM cyclin A (see Supplementary Information Fig. 2). These experiments (also see Supplementary Information Fig. 3) provided no evidence that the *Xenopus* Cdc6 protein is an inhibitor of mitotic Cdk kinases. All together, our results demonstrate that GVBD and Cdc6 synthesis are the only events that limit the development of the oocyte's replication competence during meiosis.

Our experiments define the parameters controlling the development of replication competence in *Xenopus* oocytes during meiosis. The most important parameter is Cdc6 synthesis. Without Cdc6 immature oocytes cannot assemble pre-RCs and are unable to replicate DNA. The subcellular localization of replication factors is another parameter controlling replication competence in *Xenopus* oocytes. In immature G2-arrested oocytes, Orc proteins are cytoplasmic and therefore unable to support pre-RC assembly. This is the first report of nuclear exclusion of the Orc proteins during a G2 arrest. It will be important to determine the mechanisms responsible for the cytoplasmic retention of Orc and if these mechanisms are meiosis-specific or also involved in controlling pre-RC assembly during the mitotic cell cycle. The same questions should be asked regarding the cytoplasmic localization of the Cdc7 kinase that has been described as a nuclear-localized protein during the somatic cell cycle^{18,19}. Although additional parameters (for example, Cdk-dependent phosphorylation of Mcm4 in the GV) might contribute to the initiation defect of immature oocytes, the extent of any such additional mechanisms remains to be established. We have shown that replication competence in *Xenopus* oocytes is established during meiosis I after Cdc6 synthesis and nuclear envelope breakdown. Once replication competence is established, DNA replication has to be repressed until meiosis is completed. Now the challenge is to identify the mechanisms acting downstream of Mos and MPF that are responsible for this repression. □

Methods

Oocyte treatments

Xenopus oocytes were prepared, cultured and microinjected as described³. During maturation kinetics, oocytes were pooled and synchronized at GVBD. The time of GVBD was determined by the appearance of a white spot on the animal pole. For western blot analysis, ten oocytes were homogenized in 100 µl of extract buffer (EB; 20 mM K-HEPES pH 7.6, 200 mM KCl, 3 mM MgCl₂, 5 mM EGTA, 80 mM β-glycerophosphate, 10 mM sodium fluoride, 1 mM sodium vanadate and 10 µg ml⁻¹ aprotinin, leupeptin and pepstatin). Extracts were centrifuged at 4 °C for 10 min and 20,000g. The supernatant equivalent to 1.5 to 3 oocytes was analysed by SDS-polyacrylamide gel electrophoresis (PAGE). The same protocol was used to prepare cytoplasmic or GV extracts following the manual enucleation of stage-VI oocytes. Low-speed extract of immature oocytes²⁰ was used to determine pre-RC assembly. Binding of pre-RC components to the chromatin was performed as described¹⁰.

Recombinant proteins

Xenopus Cdc6 and Cdc45 proteins were obtained using baculovirus expression constructs previously described^{21,22}. *Xenopus* cyclin A1 complementary DNA was subcloned into pFastBac Hta and a recombinant baculovirus was obtained by standard procedures (Gibco-BRL). All recombinant proteins were produced in Sf9 cells and purified using a Ni-NTA column (Qiagen) according to the manufacturer's instructions. The purity of the Cdc6 protein used for injection is greater than 90% (see Supplementary Information Fig. 4).

Antibodies

Polyclonal antibodies against *Xenopus* Cdc6, Mcm4, Cdt1, Cdc45 and Cdc7 were raised in rabbits using *E. coli* His-tag recombinant proteins as antigens. Orc1, Orc2, Mcm2, Mcm3, Mcm6, Mcm7, Cdc25 and cyclin B1 antibodies were previously described^{23–28}.

Analysis of DNA synthesis and H1 kinase assays

For replication assay maturing oocytes were injected at GVBD with a 50-nl solution containing [α -³²P]dCTP (3,000 Ci mmol; 40 mCi ml⁻¹) with or without the indicated

recombinant proteins. Oocytes were collected 4 h after GVBD and DNA was extracted. DNA corresponding to 20 oocytes was loaded on a 1% agarose gel, electrophoresed and processed for autoradiography. Under these conditions the sheared chromosomal DNA runs on agarose gel as a band of 50 kilobase (kb). H1 kinase assays were carried as described³. H1 kinase activity associated with the Cdc2/cyclinB complex present in the nucleus of immature oocytes treated with or without 200 nM of leptomycin B was determined after the Cdc2/cyclinB complex of 20 oocyte nuclei was isolated on p13^{suc1} beads.

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The DIX domain targets dishevelled to actin stress fibres and vesicular membranes

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Colorectal cancer results from mutations in components of the Wnt pathway that regulate β -catenin levels¹. Dishevelled (Dvl or Dsh) signals downstream of Wnt receptors and stabilizes β -catenin during cell proliferation¹ and embryonic axis formation². Moreover, Dvl contributes to cytoskeletal reorganization during gastrulation^{3–5} and mitotic spindle orientation during asymmetric cell division⁶. Dvl belongs to a family of eukaryotic signalling proteins that contain a conserved 85-residue module of unknown structure and biological function called the DIX domain⁷. Here we show that the DIX domain mediates targeting to actin stress fibres and cytoplasmic vesicles *in vivo*. Neighbouring interaction sites for actin and phospholipid are identified between two helices by nuclear magnetic resonance spectroscopy (NMR). Mutation of the actin-binding motif abolishes the cytoskeletal localization of Dvl, but enhances Wnt/ β -catenin signalling and axis induction in *Xenopus*. By contrast, mutation of the phospholipid interaction site disrupts vesicular association of Dvl, Dvl phosphorylation, and Wnt/ β -catenin pathway activation. We propose that partitioning of Dvl into cytoskeletal and vesicular pools by the DIX domain represents a point of divergence in Wnt signalling.

Cell proliferation and morphogenesis involve subcellular redistributions of signalling proteins such as Dvl^{4–6}. On Wnt stimulation of embryonic kidney cells, endogenous Dvl migrates to the perinuclear region where the signalling machinery resides, and also localizes to actin stress fibres⁸. These contractile bundles of actin filaments are anchored to the substratum by focal adhesion plaques, and their dynamics underlie cell motility⁹. Consistent with these observations, we found that endogenous dishevelled-2 (Dvl2) co-localizes with sets of actin stress fibres (Fig. 1b) that span Chinese hamster ovary K1 (CHO) cells. Dvl2 also exhibits a punctate pattern, suggesting some distribution to cytoplasmic vesicles. The identity of these Dvl-associated vesicles in a variety of cell types has remained unclear since their discovery¹⁰. They may be endosomal, Golgi-derived, or transport vesicles as indicated by double labelling with GAP-43 (ref. 11), a protein that also co-localizes with another β -catenin regulator, glycogen synthase kinase-3 β ¹². However, RhoB, a marker for early endosomes and prelysosomal compartments¹³, does not co-stain with Dvl2 (ref. 14). In *Xenopus* embryos, Dvl associates with 0.3–0.5- μ m diameter vesicles that appear to transport Dvl towards the prospective dorsal side of the embryo during cortical rotation¹⁵.

To establish whether the DIX domain is responsible for Dvl targeting, the localization of Dvl2 constructs (Fig. 1a) was investigated in CHO cells, which exhibit well-characterized Wnt responses¹⁶. Similar to endogenous Dvl2, the overexpressed full-

length protein co-localized with actin stress fibres (Fig. 1c), as did its isolated DIX domain (Fig. 1d). In contrast, a construct lacking the DIX domain (Dvl2 Δ DIX) was diffusely distributed in the cytosol (Fig. 1e). Similarly axin, a protein that destabilizes β -catenin⁷,

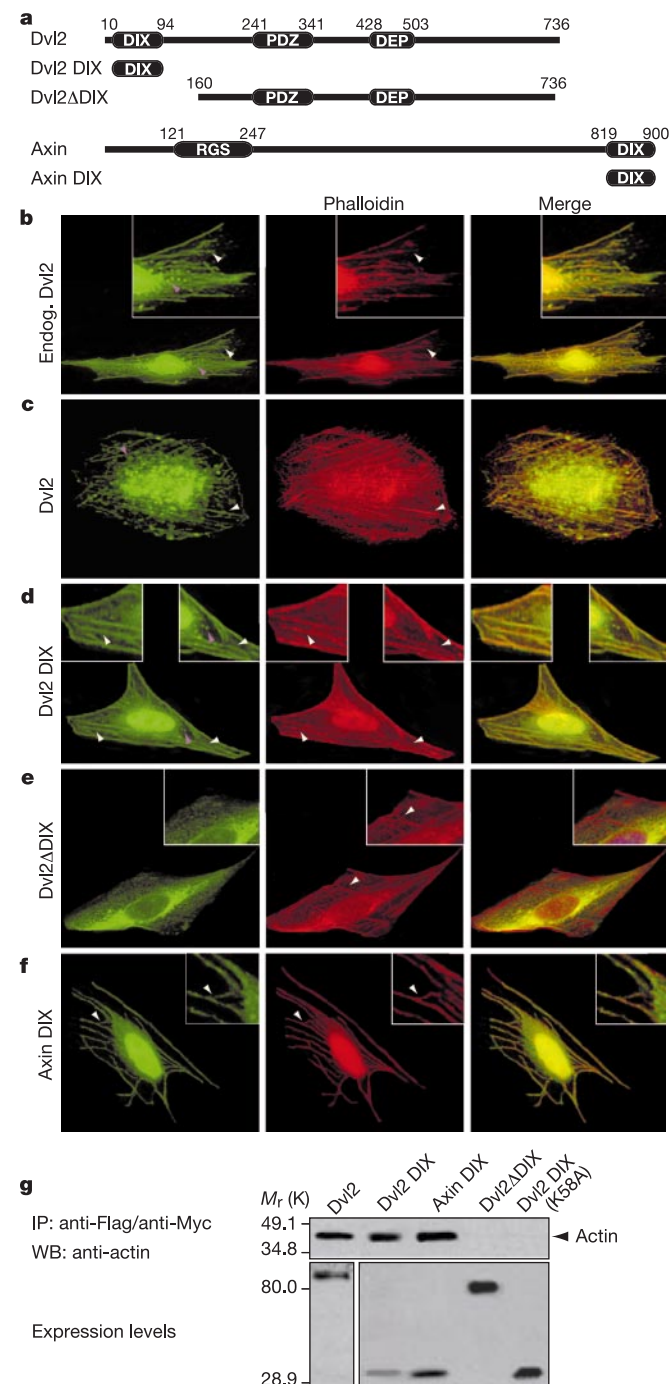


Figure 1 The DIX domain associates with actin. **a**, Architecture of the constructs. **b**, Endogenous Dvl2 was immunostained in CHO cells with anti-Dvl2 antibody (green). Phalloidin (red) stains actin stress fibres (white arrowheads). Pink arrowheads indicate cytoplasmic punctations. The merged image reveals the co-localization of Dvl and actin (yellow); 0.4 cm represents approximately 10 μ m. **c–f**, Transfected CHO cells were immunostained. **c**, Full-length Dvl2 localizes to actin stress fibres and cytoplasmic punctations. **d, e**, The Dvl2 DIX domain associates with actin stress fibres (**d**), whereas the Dvl2 Δ DIX mutant does not (**e**). **f**, The axin DIX domain localizes to actin. Insets show greater magnification of main images. **g**, Actin is detected with immunoprecipitated Dvl2 and axin constructs containing wild-type DIX domains but not DIX deletions or K58A mutations. Dvl2 (Flag-tagged) and axin (Myc-tagged) expression levels were determined by immunoblotting. IP, immunoprecipitation; WB, western blot.

possesses a DIX domain that also co-localizes with stress fibres (Fig. 1f). Furthermore, endogenous actin immunoprecipitated with full-length Dvl2 and the isolated DIX domains of Dvl2 and axin, but not the highly expressed Dvl2 Δ DIX protein (Fig. 1g). Thus the Dvl2 DIX domain is necessary and sufficient for actin stress fibre association, and this DIX function seems to be conserved.

DIX domains are known to mediate oligomerization of Dvl and axin^{14,17,18}, as confirmed here by pull-down experiments (Supplementary Information Fig. 1a). These homo- and heteromeric complexes are also formed in the presence of dodecylphosphocholine (DPC). This detergent mimics the predominant phospholipid in mammalian membranes and forms small micelles suitable for NMR studies of membrane-associated proteins¹⁹. We determined that the DIX domain, which has a relative molecular mass of 9,800 (M_r , 9.8K), exists as a homodimer of approximately 21.2K by pulsed-field gradient NMR studies (Supplementary Information Fig. 1b). This homodimeric state was confirmed by the protein's narrow NMR lines and the appearance of an approximately 21.5K species after incubation with the bis(sulphosuccinimidyl) suberate³ crosslinker (Supplementary Information Fig. 1c).

The Dvl2 DIX domain forms a predominantly helical structure. The helical content of the DIX domain rose to 26% when DPC micelles were added based on circular dichroism spectroscopy (Supplementary Information Fig. 2a and Table 1). NMR studies predict two α -helices spanning residues Ala 35 to Gln 48 (α 1) and Ala 51 to Tyr 55 (α 2), and helical and extended elements from Asn 82 to Leu 89 (α 3) and Val 29 to Ile 31, respectively (Supplementary Information Fig. 2c). However, the paucity of long-range nuclear Overhauser effects precluded determination of the three-dimensional solution structure.

An actin-binding element was identified between the α 2 and α 3 helices. Addition of monomeric actin to the ¹⁵N-labelled Dvl2 DIX domain broadens the protein's [¹H, ¹⁵N] NMR signals, especially in and around α 2, suggesting formation of a large, slowly tumbling

complex. Despite the extensive line-broadening, progressive chemical shift perturbations were detected in a variety of basic and hydrophobic residues, indicating their direct or indirect involvement in actin binding (Supplementary Information Fig. 3). The amide resonances of Lys 58, Ser 59 and Met 60 register the most pronounced changes, suggesting that these residues are critically involved in the actin interaction. These residues lie in the conserved YFFKSM-60 sequence (Supplementary Information Fig. 2d), which shares hydrophobic and basic character with an established actin-binding motif represented by FSFKKS and V(T/H)VKKV in the MARCKS²⁰ and actobindin²¹ proteins, respectively. The most conserved residue of this motif, Lys 58, was mutated to alanine. The K58A mutation renders the DIX domain unable to bind actin by immunoprecipitation (Fig. 1g), results in a diffuse distribution (Fig. 2a), and does not compromise the structural integrity of the domain (Supplementary Information Fig. 2b and Table 2). Consequently, the YFFKSM-60 motif is functionally required for Dvl2 binding to actin stress fibres.

In addition to actin association, the Dvl2 DIX domain also appears to localize to cytoplasmic vesicles (Fig. 1b–d). The latter interaction was further investigated with concanavalin A (Con A), a lectin that binds glycoproteins in many cytoplasmic membranes. Similar to full-length Dvl2 (Fig. 1b, c), some DIX domain localizes to these cytoplasmic vesicles (Fig. 2b). By contrast, the Dvl2 Δ DIX construct, which retains the plasma-membrane-targeting DEP domain^{22,23}, was more diffusely distributed (Fig. 1e), suggesting that the DIX domain contributes to the vesicular association of Dvl2.

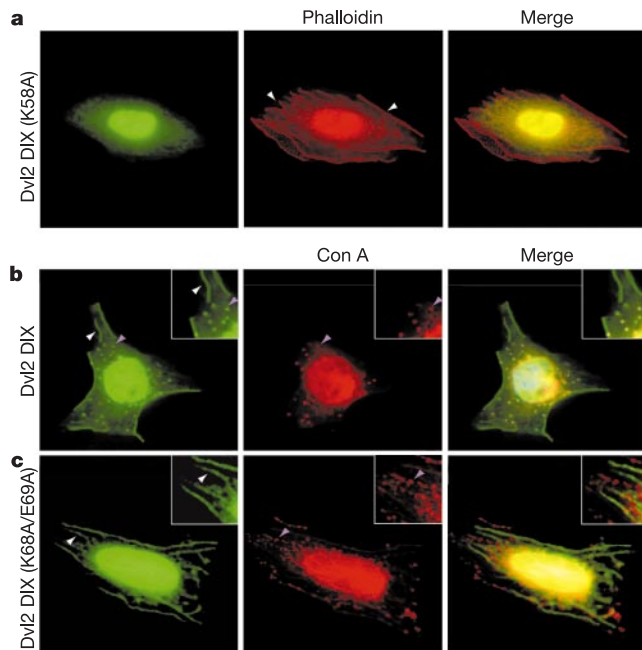


Figure 2 DIX domain motifs targeting actin and vesicles. **a**, The K58A mutant of the Dvl2 DIX domain is stained in green by the anti-Myc antibody, and does not overlap with actin stress fibres (white arrowheads), as seen in the merge. **b**, The Dvl2 DIX domain co-localizes with Con A to cytoplasmic vesicles as well as actin stress fibres in CHO cells, as indicated by pink and white arrowheads, respectively. **c**, A K68A/E69A mutant of the Dvl2 DIX domain co-localizes with actin stress fibres but not vesicles. The proteins and membranes are stained with antibody (green) and Con A (red), respectively.

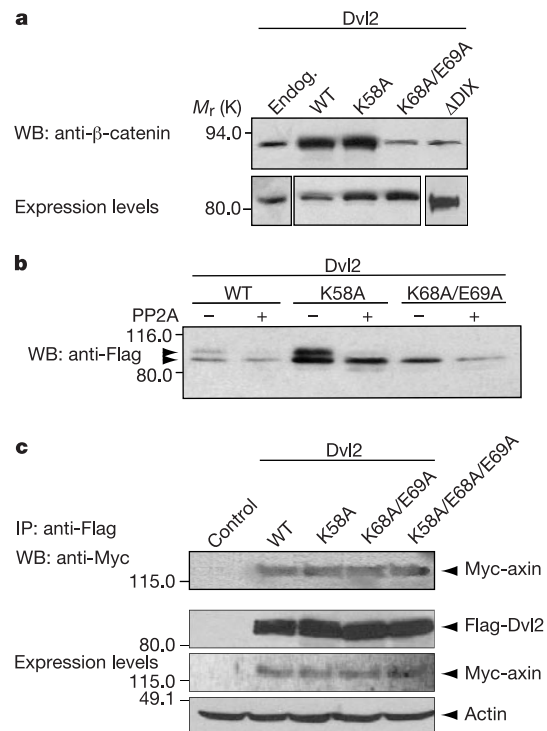


Figure 3 Cellular function of the DIX domain. **a**, Dvl2 mutations affect β -catenin levels. CHO cells were transfected with the indicated Dvl2 constructs, and the lysate was blotted with anti- β -catenin for comparison to the untransfected control (upper panel). Expression levels were detected using anti-Dvl2, anti-Flag and anti-Myc antibodies (lower panel). WT, wild type. **b**, Wild-type and Dvl2(K58A) are phosphorylated in CHO cells in a PP2A-reversible manner, whereas Dvl2(K68A/E69A) is not phosphorylated. **c**, All Dvl2 constructs bind axin comparably. *Xenopus* embryos were injected with wild-type or mutant Flag-Dvl2 and Myc-axin RNA at the single cell stage, incubated, immunoprecipitated with anti-Flag, and probed with anti-Myc antibody (upper panel). Expression levels of the Dvl2 and axin constructs were determined by immunoblotting. Loading controls were monitored with anti-actin antibody.

A phospholipid interaction loop is apparent. Titration of DPC micelles into a NMR sample of the Dvl2 DIX domain perturbs a subset of amide resonances (Supplementary Information Fig. 4). The largest changes in chemical shift are found in Glu 24, Val 67, Lys 68, Glu 69, Ile 71 and Ser 72. Thus micelle interactions primarily involve the conserved VKEEIS sequence between the principal actin-binding motif and $\alpha 3$. Notably, actin and phospholipid binding may compete, as addition of actin to the DPC-associated DIX domain reverses the DPC-induced chemical shift perturbations. Conversely, addition of DPC to the DIX-actin complex reverses the actin-induced line broadening (Supplementary Information Fig. 5). To examine these phospholipid interactions *in vivo*, the Lys 68 and Glu 69 residues of the Dvl2 DIX domain were mutated to alanines. The K68A/E69A mutant does not associate with vesicles, although actin stress fibre co-localization and struc-

tural integrity are retained (Fig. 2c and Supplementary Information Table 2). Thus the VKEEIS motif of the DIX domain mediates phospholipid interactions that recruit Dvl2 to cytoplasmic vesicles.

Stabilization of β -catenin through canonical Wnt signalling involves membrane targeting of Dvl^{14,15,23,24}. Therefore mutation of the DIX domain's functional sites could differentially affect intracellular β -catenin stability. Elevated levels of cytosolic β -catenin were found in CHO cells expressing full-length Dvl2 (Fig. 3a), consistent with the inhibition of β -catenin destruction by Dvl. The critical role of the DIX domain in Wnt regulation¹⁰ was confirmed by the inability of Dvl2 Δ DIX to stabilize β -catenin. Moreover stabilization of β -catenin is lost when the vesicle-targeting motif of Dvl2 is mutated (Dvl2(K68A/E69A); Fig. 3a). However, actin binding seems to be dispensable, as the β -catenin in cells expressing the Dvl2 K58A mutant (Dvl2(K58A)) mirrored the elevated level seen with wild-type Dvl2. Thus the DIX domain mediates formation of a vesicular pool of Dvl that stabilizes β -catenin and ensures commitment to canonical Wnt signalling.

The localization of Dvl to vesicles correlates with its regulated phosphorylation in Wnt and planar cell polarity pathways^{10,25}. Dvl phosphorylation can be catalysed by casein kinase I ϵ (ref. 26) and PAR1 (ref. 16), and can be inhibited by PP2A, a serine/threonine phosphatase²⁷. Accordingly, phosphorylated and dephosphorylated Dvl2 are found in CHO cells, with PP2A converting the former to the latter state (Fig. 3b). Similarly Dvl2(K58A), which is disabled for actin binding, is heavily phosphorylated *in vivo*. However Dvl2(K68A/E69A), which is impaired in vesicular localization, is not phosphorylated. Consequently Dvl2 phosphorylation and DIX-mediated vesicle association are linked, although a causal relationship remains to be established.

The biological implications of targeting Dvl to actin or vesicles was further examined by *Xenopus* axis induction. Endogenous β -catenin signalling activates genes that are essential for dorsal axis formation including *Xnr3* and *siamois*². Furthermore ectopic Wnt/ β -catenin signalling, such as Dvl2 overexpression, induces *Xnr3* and *siamois* expression and dorsal axis duplication². The Dvl2(K58A) mutant, which does not bind actin but expresses comparably to wild-type Dvl2 (Fig. 4a), also induces *Xnr3/siamois* expression (Fig. 4b) and boosts the axis duplication frequency (Fig. 4c, d). Thus the actin pool of Dvl is sequestered from downstream Wnt/ β -catenin signalling. By contrast, the Dvl2(K68A/E69A) mutant, which is defective in vesicle targeting, does not induce *Xnr3/siamois* expression and axis duplication (Fig. 4a–d), suggesting that vesicular Dvl2 is needed for β -catenin-dependent transcriptional programmes. Axis duplication was also not induced by a Dvl2(K58A/K68A/E69A) triple mutant that is compromised in both vesicle and actin interactions (Supplementary Information Fig. 6), emphasizing the fact that vesicular Dvl is required for Wnt signalling. Finally, axin associates comparably with each of the Dvl2 mutants when expressed in *Xenopus* embryos (Fig. 3c). Consequently these mutations specifically block vesicle and actin targeting and not axin association, which relies on several Dvl regions^{17,18}.

The DIX domain represents a novel signalling module that mediates actin stress fibre and vesicular targeting. Dvl thus partitions between these two pools, and commits to distinct pathways. This segregation is not only critical for proliferation and morphogenesis, but may also contribute to oncogenesis, with the most frequent mutations of the axin tumour suppressor found in colorectal, medullablastoma and hepatocellular tumours (ref. 28 and references therein) centred on its DIX domain. □

Methods

DNA constructs and site-directed mutagenesis

Full-length mouse Dvl2 and human axin were subcloned into pCS2+Flag and pCS2+Myc tag (MT), respectively. DNA fragments encoding Dvl2 residues 10–94 (Dvl2 DIX) and 160–736 (Dvl2 Δ DIX) and axin residues 819–900 (axin DIX) were cloned into pCS2+MT and pGEX-2T (Amersham). Mutations were generated by QuickChange (Stratagene).

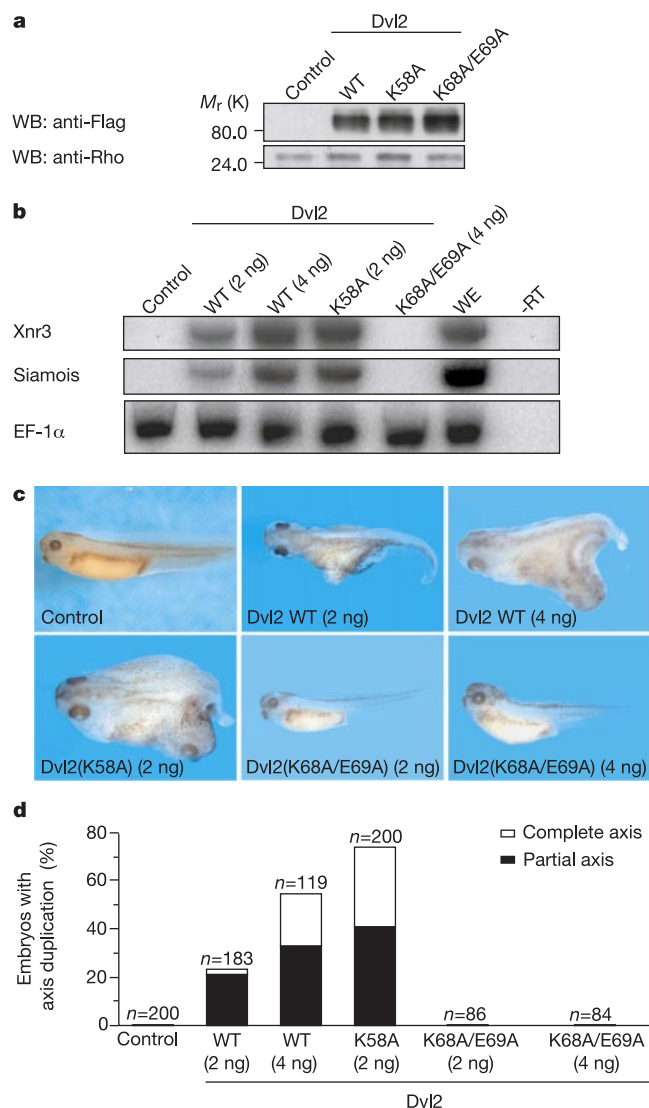


Figure 4 Biological role of the DIX domain. **a**, Expression levels of Dvl2 constructs. Whole *Xenopus* embryo extracts were made at stage 10, and immunoblotted with an anti-Flag antibody or an anti-Rho antibody. Control indicates uninjected embryos. **b**, RNA for Dvl2 or mutants was injected into the animal region at the 2-cell stage at indicated doses. Animal pole explants were excised at stage 9 and cultured until stage 10. Expression of *Xnr3* and *siamois* was examined by RT-PCR. WE, whole embryo as a positive control for RT-PCR; EF-1 α , loading control; -RT, reverse transcriptase omitted. **c**, **d**, Dvl2 RNA was injected ventrally at the 4-cell stage at indicated doses. Axis duplications were scored at stage 35. Duplicated axes with or without eyes were scored as complete or partial duplications, respectively. n, number of embryos scored.

Cell culture

CHO cells were grown in 100-mm dishes with F-12 medium (Life Technologies) supplemented with 10% fetal bovine serum. Non-synchronous cells were transiently transfected with 0.5 µg DNA per 35-mm well using Lipofectamine (Life Technologies). After 5 h, the transfection was stopped by switching to normal growth medium. Cells were collected 24 h later for immunoprecipitation or fixed for immunofluorescence.

Immunofluorescence staining

Transfected CHO cells with 70–80% confluence were fixed in 3.7% formaldehyde, 0.5% Triton X-100 in phosphate-buffered saline solution (PBS) at room temperature for 10 min. Post-extraction was performed in PBS containing 0.5% Triton X-100 for 5 min. Cells were washed in PBS with 0.1% Triton X-100 and then incubated in blocking solution (PBS with 20% normal goat serum) for 30 min. Cells transfected with Myc constructs were incubated with anti-Myc Cy3-labelled antibody, stained and fixed. Cells transfected with Flag constructs were incubated with anti-Flag (M2) and Cy3-anti-mouse antibody (Jackson Laboratories). Endogenous Dvl2 was visualized using goat anti-Dvl2 antibody (Santa Cruz) and blocking with 5% BSA instead of goat serum. Actin stress fibres and membranes were visualized using Alexa-488-phalloidin and Alexa-488-Con A (Molecular Probes), respectively. Samples were washed, mounted, analysed, and representative normal cells were selected using a fluorescence microscope equipped with a charge-coupled device camera (Nikon Eclipse TE300).

Immunoprecipitation and immunoblotting

Triton X-100-soluble aliquots from transfected CHO cells were prepared as described⁸. Actin binding was detected using goat polyclonal anti-actin antibody (Santa Cruz). The Myc- or Flag-tagged proteins were detected using anti-Myc (clone 9E10) and anti-Flag (M2) mouse monoclonal antibodies, respectively (Sigma). Primary antibodies were coupled to rabbit anti-goat (Jackson Laboratories) or horseradish peroxidase (HRP) donkey anti-mouse antibodies (Amersham). Polyvinylidene fluoride blots were then incubated with either HRP-conjugated anti-rabbit or HRP-conjugated anti-HRP antibodies (Amersham) and the signal visualized by enhanced chemiluminescence. For β-catenin detection, transfected CHO cells were lysed, and the soluble proteins were collected by centrifugation, separated by SDS–polyacrylamide gel electrophoresis, blotted, and reacted with anti-β-catenin antibody (Transduction Laboratories).

Phosphatase treatment

Triton X-100-soluble aliquots (5 µl) of Dvl2-transfected CHO cells were incubated at 30 °C for 1 h with 0.05 U of PP2A (Upstate) in 20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 60 mM β-mercapthoethanol, 1 mM MgCl₂, 2 mM EGTA, 100 µM MnCl₂ and 100 µg ml⁻¹ BSA. Reactions were stopped by the addition of SDS sample buffer, resolved in Anderson gels²⁹, transferred and detected by western blotting.

Xenopus assays

In vitro RNA synthesis, *Xenopus laevis* embryo staging, injection, animal pole explants, polymerase chain reaction with reverse transcription (RT–PCR) and embryo extracts were performed as described³⁰.

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Myc suppression of the *p21^{Cip1}* Cdk inhibitor influences the outcome of the p53 response to DNA damage

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Activation of the tumour suppressor p53 by DNA damage induces either cell cycle arrest or apoptotic cell death¹. The cytostatic effect of p53 is mediated by transcriptional activation of the cyclin-dependent kinase (CDK) inhibitor *p21^{Cip1}*, whereas the apoptotic effect is mediated by transcriptional activation of mediators including *PUMA* and *PIG3* (ref. 2). What determines the choice between cytosclerosis and apoptosis is not clear³. Here we show that the transcription factor Myc is a principal determinant of this choice. Myc is directly recruited to the *p21^{Cip1}* promoter by the DNA-binding protein Miz-1. This interaction blocks *p21^{Cip1}* induction by p53 and other activators. As a result Myc switches, from cytosclerosis to apoptotic, the p53-dependent

response of colon cancer cells to DNA damage. Myc does not modify the ability of p53 to bind to the *p21^{Cip1}* or *PUMA* promoters, but selectively inhibits bound p53 from activating *p21^{Cip1}* transcription. By inhibiting *p21^{Cip1}* expression Myc favours the initiation of apoptosis, thereby influencing the outcome of a p53 response in favour of cell death.

Myc is a transcription factor that promotes cell growth and proliferation, and, under certain conditions, apoptosis⁴. Levels of Myc are increased or decreased in response to mitogenic or growth-inhibitory stimuli, respectively^{4,5}. In association with the protein Max, Myc binds to a specific DNA sequence, the E box, and orchestrates transcriptional activation of a diverse set of genes⁴. On other genes, including *p21^{Cip1}* (refs 6, 7) and another cyclin-dependent kinase inhibitor, *p15^{Ink4b}* (ref. 8), Myc inhibits transcription.

Diverse signals, including the antimitogenic cytokine transforming growth factor- β (TGF- β)⁹, p53 (ref. 10) and the protein kinase C activator phorbol ester tetradecanoyl-phorbol acetate (TPA)¹¹, can activate *p21^{Cip1}* transcription. In the spontaneously immortalized human keratinocyte line HaCaT, addition of TGF- β induces accumulation of p21^{Cip1} with rapid and sustained kinetics, whereas TPA induces a weaker and more transient response (Fig. 1a). To investigate the effect of Myc on the expression of p21^{Cip1}, we created a HaCaT derivative, HaCaT-tetMyc, with a human *myc* transgene under the control of the *tet* regulator. Placing these cells in tetracycline-free medium leads to an accumulation of Myc of up to threefold over the endogenous level (Fig. 1b). Although limited, this increase was sufficient to inhibit the induction of p21^{Cip1} by TGF- β or TPA (Fig. 1b). Tetracycline had no effect on Myc or p21^{Cip1} levels in control cells lacking the *myc* transgene (data not shown).

TGF- β caused an abrupt downregulation of endogenous Myc in HaCaT cells (Fig. 1b) but TPA did not (Fig. 1b). Thus, the level of endogenous Myc in exponentially growing cells allows at least a sub-maximal accumulation of p21^{Cip1}, but a threefold increase in Myc levels, as in HaCaT-tetMyc cells, does not. Of note, these results suggest that Myc can interfere with p21^{Cip1} induction by mechanistically distinct activators. TGF- β regulates gene expression through Smad transcription factors¹², whereas TPA is thought to act through protein kinase C and Sp1 or AP2 transcription factors^{13,14}. This led us to investigate whether Myc affects the induction of p21^{Cip1} by yet another activator, p53.

As a model for studies on p53, we used the human colon carcinoma cell line HCT116 and isogenic derivatives from this cell line in which *p21^{Cip1}* or *p53* had been deleted by homologous recombination^{15,16}. Daunorubicin is a chemotherapeutic anthracycline that creates DNA breaks by inhibiting topoisomerase II during DNA replication¹⁷. Daunorubicin induces accumulation of p21^{Cip1} in HCT116 cells in a p53-dependent manner (Fig. 1a). Infection of HCT116 cells with adenovirus encoding Myc did not prevent the elevation of p53 levels by daunorubicin, but strongly inhibited the induction of p21^{Cip1} (Fig. 1b). This effect was also apparent when p21^{Cip1} expression was determined using immunofluorescence (Fig. 1c). Very few of the cells overexpressing Myc showed an induction of p21^{Cip1} by daunorubicin, in contrast to the induction of p21^{Cip1} seen in a large proportion of untransfected cells (Fig. 1c, d). Similar results were obtained when the effect of Myc on p21^{Cip1} expression was determined by immunofluorescence in HaCaT cells treated with TGF- β (Fig. 1e).

In transcriptional reporter assays, activation of the *p21^{Cip1}* promoter by daunorubicin addition or by p53 overexpression was

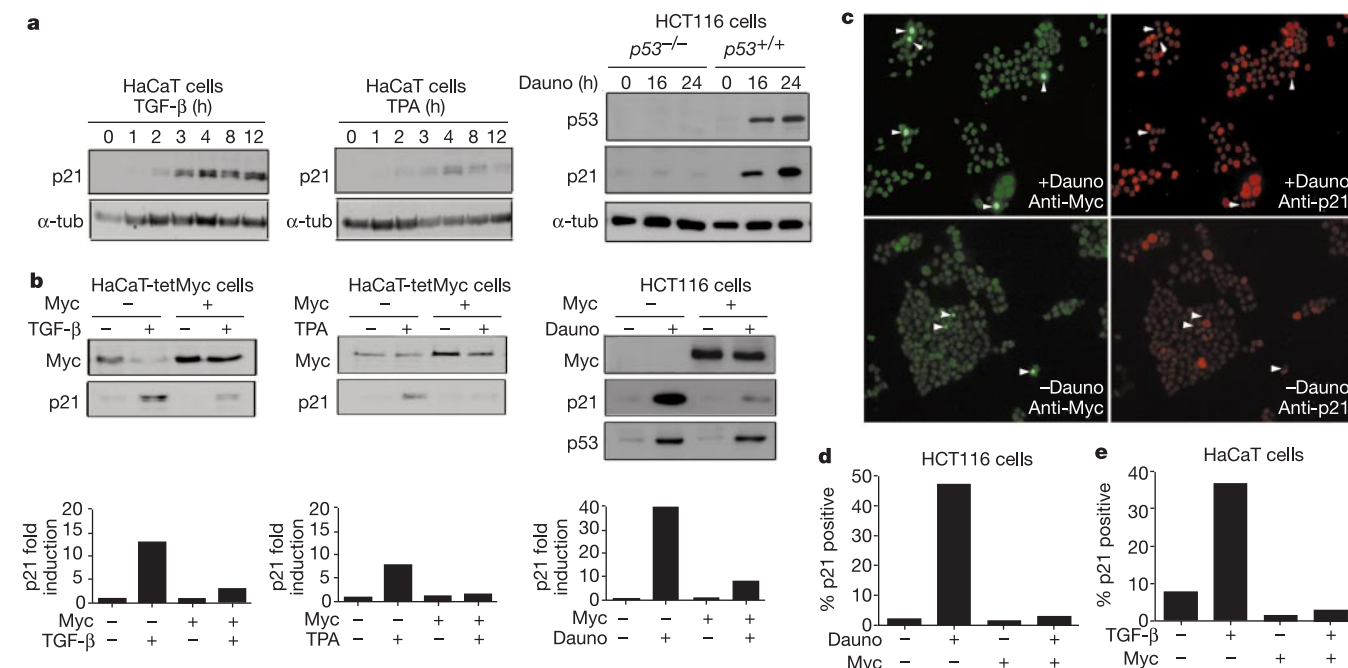


Figure 1 Overexpression of Myc inhibits p21 induction by TGF- β , TPA and p53. **a**, HaCaT cells were incubated with TGF- β or TPA for the specified times. HCT116 (*p53*^{+/+} and *p53*^{-/-}) cells were incubated in the presence or absence of daunorubicin (Dauno) as indicated. Cell extracts were subjected to immunoblot analysis for p21, p53 or α -tubulin (α -tub) expression. **b**, HaCaT-tetMyc cells were cultured for 20 h with or without tetracycline, followed by treatment with either TGF- β or TPA for a further 4 h, and protein lysates were subjected to immunoblot analysis. HCT116 cells were infected with Ad-Myc-GFP and treated with daunorubicin for 18 h before performing an immunoblot analysis. **c**, Myc (green) and p21 (red) expression detected by immunofluorescence in HCT116 cells transfected with an Myc expression plasmid and incubated in the presence or

absence of daunorubicin for 18 h. Arrowheads, cells overexpressing Myc. **d**, Cells from the immunofluorescence assay in **c** were scored as p21-positive (bright red) or p21-negative from the four indicated groups: Myc-negative cells in the absence of daunorubicin; Myc-negative cells treated with daunorubicin; Myc-positive (bright green) cells in the absence of daunorubicin; and Myc-positive (bright green) cells treated with daunorubicin. The graph represents the percentage of p21 positive cells determined from 50 cells in each group. **e**, HaCaT cells transfected with an Myc expression plasmid and incubated in the presence or absence of TGF- β for 8 h. Myc (green) and p21 (red) expression were detected by immunofluorescence as in **c**, and the percentage of p21 positive cells was determined as in **d**.

inhibited by overexpression of Myc (Fig. 2a). To map the region of *p21^{Cip1}* conferring susceptibility to inhibition of this promoter by Myc, we used TGF- β as the activator. The use of p53 in this experiment was precluded, as 5' promoter deletions eliminate the p53 response sites of *p21^{Cip1}*, which are located at positions -1395 and -2281 (ref. 18). The region conferring susceptibility to *p21^{Cip1}* inhibition by Myc mapped to the proximal promoter region (Fig. 2b), in agreement with previous observations⁷. Overexpression of Myc did not inhibit the activation of a reporter, 4 $\times$ SBE¹⁹, which contains a multimerized Smad-binding element (Fig. 2b), indicating that Myc does not act by directly interfering with Smads.

We recently elucidated the mechanism of Myc inhibition of *p15^{Ink4b}* expression^{20,21}. The DNA-binding factor Miz-1 (Myc-interacting zinc-finger 1) recruits Myc to the transcriptional initiator region (Inr) of the *p15^{Ink4b}* promoter, interfering with recruitment of transcriptional co-activators^{20,21}. Unlike the *p15^{Ink4b}* promoter, however, the *p21^{Cip1}* promoter contains a TATA box, and has an overall distinct topology. Nonetheless, chromatin immunoprecipitation (ChIP) assays demonstrated that both Myc and Miz-1 associate with the proximal promoter region of *p21^{Cip1}* *in vivo*, as they do with *p15^{Ink4b}* (Fig. 2c). No Myc or Miz-1 binding was detected on a *p21^{Cip1}* distal promoter region or on the β -actin promoter, used as negative controls. Myc, but not Miz-1, was detected on the prothymosin promoter, an activation target of Myc that contains a canonical E box (Fig. 2c). Thus, Miz-1 and Myc can target the *p21^{Cip1}* promoter for inhibition even though the sequence and topology of this promoter do not conform to the TATA-less and transcriptional initiator region configuration of previously known Myc/Miz-1 inhibited genes.

Purified Miz-1 protein contacted two discrete sites—site A (-46 to -32 (-46/-32)) and B (-105/-81)—on the *p21^{Cip1}* proximal promoter region (Fig. 2d). Site A includes the TATA box, whereas site B, located further upstream, lies between two *Sp1* sites. Neither region has sequence similarity to the Miz-1 contact regions (-1/+13 Inr and -155/-140) of the *p15^{Ink4b}* promoter (Fig. 2d) or low-density lipoprotein receptor promoter sequences that bind Miz-1 for transcriptional activation²². As Miz-1 has 13 zinc-finger motifs, it is possible that different subsets of fingers mediate recognition of these different DNA sequences.

Precipitation assays using biotinylated double-stranded oligonucleotide probes showed that Miz-1 and Myc bind to the site A region of *p21^{Cip1}* (Fig. 2e). Myc binds to this probe in the presence of wild-type Miz-1 but not in its absence or in the presence of Miz-1 dZF, a Miz-1 deletion construct lacking the zinc-finger region²¹ (Fig. 2e). Miz-1 binding to DNA was enhanced by Myc. Similar results were obtained using a *p21^{Cip1}* probe (-122/-62) that includes site B (data not shown). Collectively these results show that Miz-1 can recruit Myc to the proximal region of the *p21^{Cip1}* promoter.

Having established *p21^{Cip1}* as a new target of inhibition by the Myc-Miz-1 complex, we investigated the hypothesis that Myc may sensitize cells to p53-dependent apoptosis by preventing expression of *p21^{Cip1}*. When expressed at high levels, Myc itself can activate p53 through p14/ARF and induce apoptosis²³. However, HCT116 cells have an inactivated *p14/ARF* locus²⁴ and so overexpression of Myc does not increase the level of p53 in these cells (see Fig. 1b). In HCT116 cells, therefore, the effect of Myc on the expression of *p21^{Cip1}* is dissociated from the effects of Myc on p53.

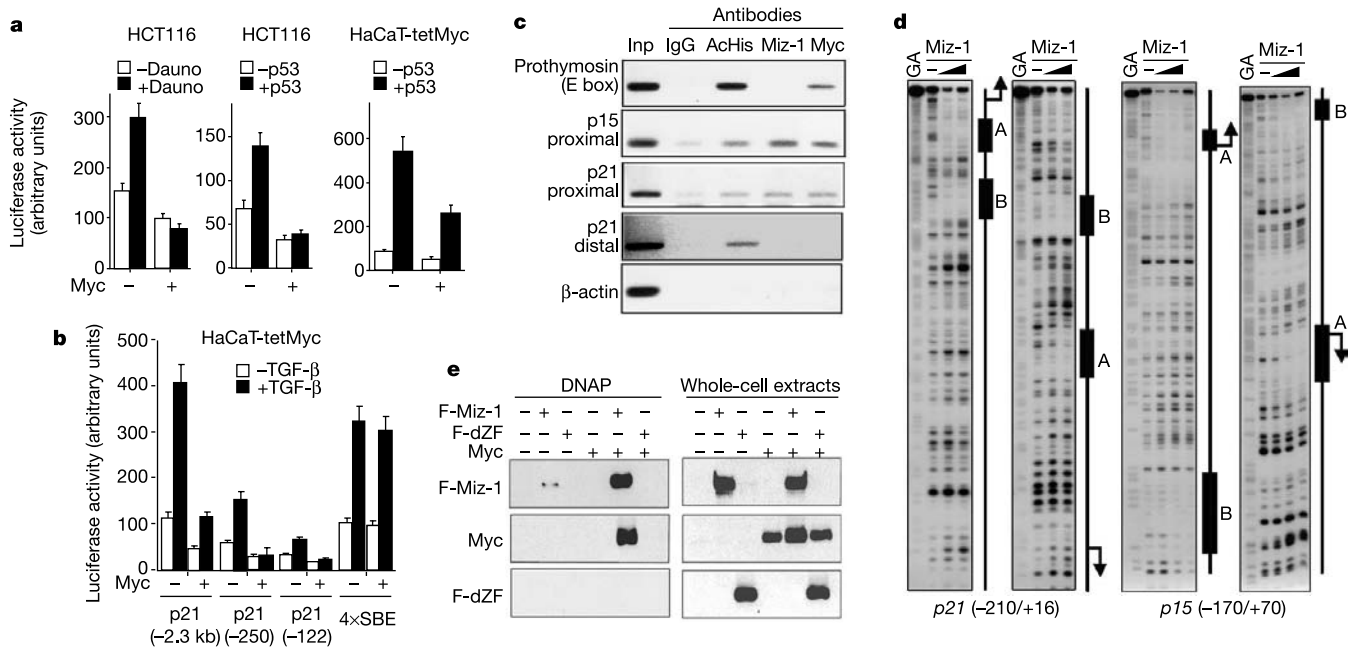


Figure 2 Myc represses *p21* induction by binding to the *p21* proximal promoter region by means of Miz-1. **a**, HCT116 cells were co-transfected with a reporter construct containing the full-length *p21* promoter (2.3 kb) with or without an Myc expression vector, and incubated in the presence or absence of daunorubicin for 20 h, as indicated. Furthermore, HCT116 cells were transfected with the full-length *p21* reporter construct, with or without a p53 expression vector and a Myc expression vector, as specified. HaCaT-tetMyc cells were transfected with the full-length *p21* reporter construct with or without a p53 expression vector, and cultured in the presence or absence of tetracycline. After treatments, cells were collected and luciferase activity was measured. **b**, HaCaT-tetMyc cells were transfected with the indicated reporter constructs, cultured for 20 h in the presence or absence of tetracycline, followed by an additional 20 h of treatment with or without TGF- β , and luciferase activity was determined. **c**, Chromatin immunoprecipitation

was carried out with HaCaT cells using the indicated antibodies. PCR was performed using primers for the indicated promoter regions. AcHis, acetylated histone. Inp, input. **d**, Footprinting experiments were conducted by incubating a ³²P-end-labelled probe containing the -210 to +16 region of the *p21* promoter or the -170 to +70 region of the *p15* promoter with increasing amounts of purified recombinant Miz-1, and subjected to Dnase I treatment. The GA lane is a size marker. Black boxes indicate the Miz-1 contact regions (see text). **e**, COS-1 cells were transfected with Myc, Flag-tagged Miz-1 (F-Miz-1), and Flag-tagged Miz-1 lacking the zinc-finger domain (F-dZF) expression vectors, as indicated. Cell extracts were precipitated with biotinylated oligonucleotides spanning the -61 bp to +16 bp of the *p21* promoter, and bound complexes were subjected to immunoblot analysis with antibodies against Flag and Myc. DNAP, DNA precipitation assay.

Daunorubicin and γ -radiation arrest HCT116 cells in G1 (2N DNA content) and G2 (4N DNA content) stages of the cell cycle, with little evidence of DNA fragmentation indicative of cell death (sub-G1 DNA content)²⁵ (Fig. 3a). Overexpression of Myc had little effect on the basal cell cycle distribution of HCT116 cells (Fig. 3a, Ad-Myc panels); however, the Myc-overexpressing cells showed a blunted $p21^{Cip1}$ response to daunorubicin or γ -radiation (Fig. 3b), and underwent massive DNA fragmentation and death (Fig. 3a). Fragmentation of poly(ADP-ribose) polymerase (PARP), a marker of caspase-mediated proteolysis during the apoptotic response, was observed after daunorubicin treatment in cells infected with adenovirus encoding Myc (Ad-Myc) but not in cells infected with control vector (Fig. 3c). Daunorubicin induced apoptosis in HCT116 cells lacking $p21^{Cip1}$ (Fig. 3a). The sub-G1 peak in this case was not as pronounced as in the case of Myc overexpression in parental HCT116 cells. Overexpression of Myc did increase this level in HCT116 $p21^{-/-}$ cells (Fig. 3a), suggesting that Myc can additionally induce apoptosis by mechanisms independent of $p21^{Cip1}$ suppression.

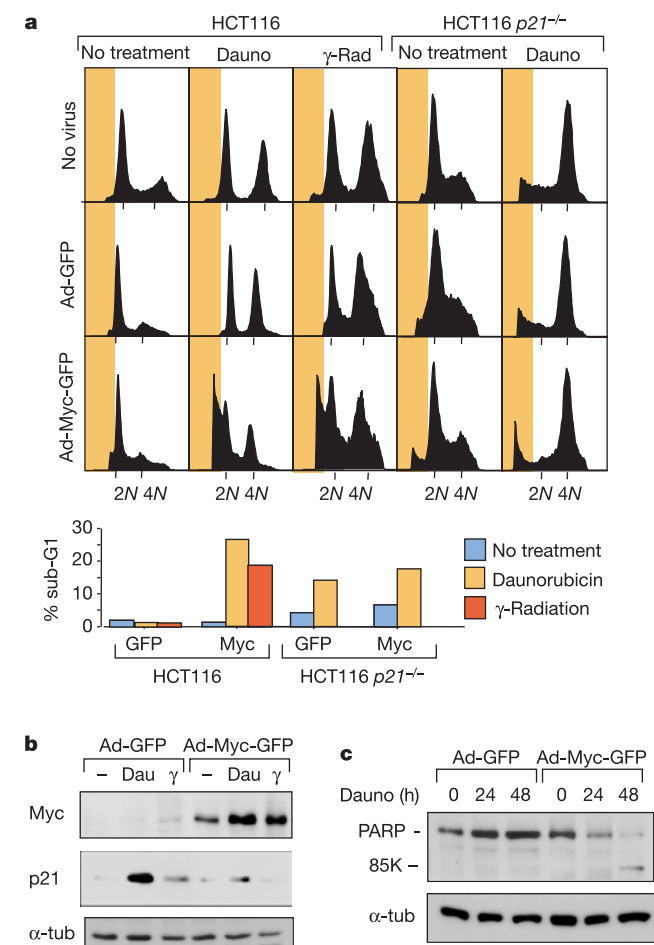


Figure 3 Myc overexpression or $p21$ loss favours apoptosis instead of cell cycle arrest on treatment with daunorubicin or γ -radiation in HCT116 cells. **a**, HCT116 cells or HCT116 $p21^{-/-}$ cells were infected with adenoviruses encoding GFP alone (Ad-GFP) as a control or with c-myc (Ad-Myc-GFP) as indicated. Cells were treated with daunorubicin or γ -radiation. DNA content was analysed 48 h later by flow cytometry of cells stained with propidium iodide. Each plot represents the analysis of 20,000 events. The colour strips in the plot mark the sub-G1 region. Quantification of the percentage of the sub-G1 DNA content was determined for the plots and represented in a graph. **b**, HCT116 cells infected with Ad-GFP or Ad-Myc-GFP as indicated were treated with daunorubicin or γ -radiation. Forty-eight hours after treatment, cell extracts were subjected to immunoblot analysis for Myc, p21 and α -tubulin. **c**, HCT116 cells infected with Ad-GFP or Ad-Myc-GFP, as indicated, were treated with daunorubicin for the specified times. Cell extracts were subjected to immunoblot analysis for PARP and α -tubulin.

tosis by mechanisms independent of $p21^{Cip1}$ suppression.

Overexpression of $p21^{Cip1}$ (Fig. 4a) caused G1 arrest and prevented daunorubicin-induced apoptosis in Myc-overexpressing HCT116 cells (Fig. 4b). We also used the colorectal cancer cell line LoVo, which is wild type for $p53$ and undergoes apoptosis on DNA damage²⁵. Daunorubicin induced $p21^{Cip1}$ expression in LoVo cells, but this level of $p21^{Cip1}$ was not sufficient to inhibit its target, Cdk2 (Fig. 4c). Enforced overexpression of $p21^{Cip1}$ in LoVo cells inhibited the daunorubicin-induced apoptosis (Fig. 4d, e). Collectively these results suggest that $p21^{Cip1}$ protects colon cancer cells from daunorubicin-induced apoptosis.

To delineate further how Myc favours apoptosis over cytostasis in response to DNA damage, we investigated the effect of daunorubicin on PUMA (p53 upregulated mediator of apoptosis) and PIG3 (p53-induced gene 3), which are transcriptional targets of p53 in the apoptotic response. PUMA, in particular, is a member of the 'BH3-domain-only' family of proteins that trigger mitochondrion-mediated apoptosis. Treatment of HCT116 cells with daunorubicin induced the accumulation of PUMA and PIG3 along with $p21^{Cip1}$ (Fig. 5a). Myc prevented the accumulation of $p21^{Cip1}$ but did not

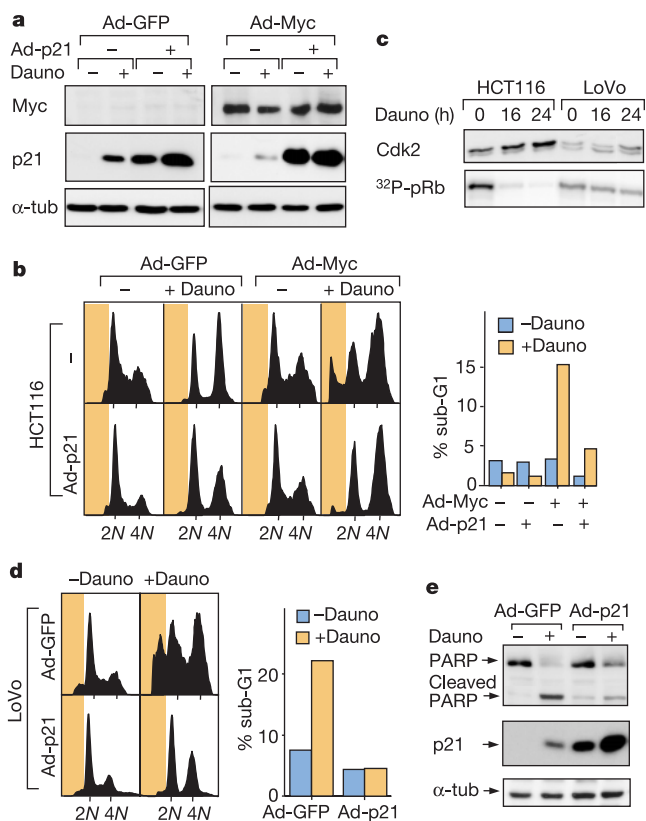


Figure 4 Effect of $p21$ overexpression on the induction of apoptosis by DNA damage in Myc-overexpressing HCT116 cells and LoVo cells. **a**, HCT116 cells infected with Ad-GFP or Ad-Myc-GFP and Ad-p21, as indicated, were treated with daunorubicin for 48 h. Cell extracts were subjected to immunoblot analysis for Myc, p21 and α -tubulin. **b**, HCT116 cells infected with Ad-GFP or Ad-Myc-GFP and Ad-p21 were treated with daunorubicin for 48 h. Flow cytometry was performed as in Fig. 3a. Quantification of the percentage of the sub-G1 DNA content was determined and is shown as a graph. **c**, HCT116 cells and LoVo cells were treated with daunorubicin for the specified times. Cell extracts were assayed for Cdk2 kinase activity. A total of 100 μ g of each cell extract was subjected to immunoblot analysis for Cdk2, pRb, retinoblastoma protein. **d**, LoVo cells infected with Ad-GFP or Ad-p21 were treated with daunorubicin for 48 h. Flow cytometry was performed as in Fig. 3a. Quantification of the percentage of the sub-G1 DNA content was determined and is shown as a graph. **e**, LoVo cells infected with Ad-GFP or Ad-p21 were treated with daunorubicin for 48 h. Cell extracts were subjected to immunoblot analysis for PARP, p21 and α -tubulin.

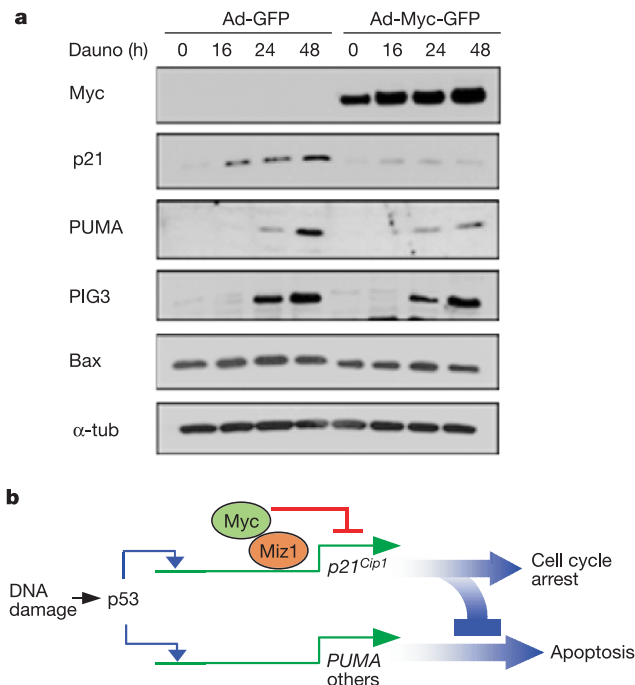


Figure 5 Effect of Myc overexpression on the induction of pro-apoptotic genes by DNA damage and on p53 DNA-binding affinity. **a**, HCT116 cells were infected with Ad-GFP or Ad-Myc-GFP as indicated, and treated with daunorubicin for the specified times. Cell extracts were subjected to immunoblot analysis for p21, PUMA, Bax, PIG3 and α -tubulin. **b**, DNA damage induces expression of p53, which in turn can induce p21^{Cip1}, leading to cell cycle arrest, or induction of PUMA and other pro-apoptotic factors, leading to cell death. When Myc levels are high, Myc is recruited to the p21^{Cip1} promoter by Miz-1, inhibiting p21^{Cip1} activation. By preventing induction of p21^{Cip1}, Myc removes p21-mediated protection against apoptosis, enabling cell death. Thus, Myc levels influence the choice between cytostasis and apoptosis induced by DNA damage.

affect the accumulation of PUMA or PIG3 (Fig. 5a). Bax, a mediator of p53-dependent apoptosis in other cell lines, was not induced by daunorubicin in these cells.

Daunorubicin increased the binding of p53 to the distal promoter region of p21^{Cip1}, which contains the p53 sites¹⁸, but not to the proximal region, which served as a control (Supplementary Fig. 1a). Daunorubicin also increased the binding of p53 to the promoter region of PUMA. When overexpressed, Myc was bound to the p21^{Cip1} proximal promoter but did not affect the binding of p53 to the p21^{Cip1} distal promoter or the PUMA promoter (Supplementary Fig. 1a). Daunorubicin induced PUMA and PIG3 accumulation in p21 null HCT116 cells as well (Supplementary Fig. 1b). These cells retain the promoter region of p21^{Cip1} (ref. 15). Daunorubicin induced p53 binding to this promoter and the PUMA promoter in p21^{-/-} cells as effectively as it did in parental HCT116 cells (Supplementary Fig. 1c).

Several conclusions can be drawn from these results. Myc selectively targets p21^{Cip1} in the p53 transcriptional programme, sparing the ability of p53 to induce the expression of PUMA or PIG3. Myc does not alter the ability of p53 to bind to the p21^{Cip1} promoter but inhibits p21^{Cip1} transcriptional activation by promoter-bound p53. In the presence of p21, p53 can still bind to the PUMA promoter and induce the accumulation of its product, but apoptosis is not achieved. Thus, the p21-dependent block in apoptosis maps to a step downstream of the DNA damage-p53-PUMA pathway. The mechanism for this provocative observation is not obvious. These results suggest a model in which Myc selectively prevents p53-dependent transcriptional activation of p21^{Cip1}, enabling pro-apoptotic factors such as PUMA to execute a cell death programme (Fig. 5b). Thus, these results define, in

mechanistic terms, how one element of the cellular context, that is, the level of Myc activity, can determine the outcome of the p53 response. Although it remains to be seen whether repression of p21^{Cip1} would be beneficial in cancer treatment, the mechanism proposed here suggests ways to influence the cell's response to stresses that result in activation of p53. □

Methods

Cell lines and cell culture

HaCaT keratinocytes and COS-1 cells were maintained in DMEM supplemented with 10% FBS and antibiotics. HCT116 wild-type, p21^{-/-} and p53^{-/-} cells were maintained in McCoy's 5A medium supplemented with 10% FBS and antibiotics. LoVo cells were maintained in RPMI medium supplemented with 10% FBS and antibiotics. The HaCaT-tTA cell line was obtained by stably transfecting the tTA vector into HaCaT cells, and stable clones were isolated after 2–3 weeks of G418 (500 μ g ml⁻¹) selection as previously described²⁶. The human myc complementary DNA was cloned into the XbaI site of the pUHD10-3 hygromycin vector. The pUHD10-3-Myc construct was transfected into the HaCaT-tTA cell line using Lipofectamine (Invitrogen) according to manufacturer's instructions. Transfected HaCaT-tTA cells were cultured under hygromycin selection (300 μ g ml⁻¹) for 2–3 weeks, at which time stable clones were isolated²⁶ to obtain the HaCaT-tetMyc cell line.

Plasmids, reagents and antibodies

Mammalian expression vectors encoding c-Myc, Flag-Miz-1, Flag-dZF2^{21,27}, p53, and the reporter constructs p21(–2.3 kb/+16)Luc, p21(–122/+16)Luc¹⁰, p15(–113/+70)Luc²⁸ and 4 × SBELuc¹⁹ have been described. The –210 to +16 region of the human p21 promoter and the –170 to +70 region of the human p15 promoter were amplified by polymerase chain reaction (PCR) and inserted into the BglII/HindIII sites of pGL2-basic (Promega) to generate p21(–210/+16)Luc and p15(–170/+70)Luc, respectively. The bacterial expression vector encoding full-length Miz-1 has been described²². Daunorubicin (Oncogene Research Products), TPA (Sigma) and TGF- β (R&D Systems) were used at a concentration of 0.5 μ M, 50 ng ml⁻¹ and 100 pM, respectively. γ -Radiation (20 Gy) was delivered by a ¹³⁷Cs gamma radiator at 2.5 Gy min⁻¹. Antibodies against Myc (9E10, Santa Cruz Biotechnology), p21 (BD Pharmingen), p53 (Pab 1801, Santa Cruz Biotechnology), α -tubulin (Sigma), M2-Flag (Sigma), Bax (N-20, Santa Cruz Biotechnology), PIG3 (Oncogene Research Products), and PARP (BD Pharmingen) were used for immunoblotting. Antibodies against Myc (N-262X, Santa Cruz Biotechnology), Miz-1 (ref. 22), p53 (Pab 1801, Santa Cruz Biotechnology) and acetylated histone H4 (Upstate) were used for chromatin immunoprecipitations.

Reporter assays

One day after seeding, HCT116 or HaCaT-tetMyc cells were transfected using Lipofectamine (Invitrogen) according to the manufacturer's instructions. After transfection, cells were treated with daunorubicin or TGF- β for 20 h. Luciferase assays were carried out using the Promega luciferase assay kit and a Berthold luminometer. A cytomegalovirus (CMV)-renilla luciferase plasmid (Promega) was included to control for transfection efficiency. Normalized values are reported as the mean $\pm$ s.d. from triplicate transfections.

Adenoviral infections

Adenovirus Ad-green fluorescent protein (GFP), Ad-p21 and Ad-Myc-GFP were amplified and titrated. Cells were grown to 70% confluence and infected with recombinant adenovirus at a multiplicity of infection (MOI) of 20 for 3 h. Twenty-four hours after the infection, cells were treated with daunorubicin for the specified times.

Oligonucleotide precipitation

Precipitation of biotinylated oligonucleotides was performed as previously described²⁹ using COS-1 cell extracts overexpressing the indicated constructs.

Footprint assay

To generate the p21 and p15 probes, p21(–210/+16)Luc or p15(–170/+70)Luc was linearized with BglII, treated with CIP (calf intestinal phosphatase), labelled at the 5' end of the sense strand with [γ -³²P]ATP using T4 polynucleotide kinase (New England BioLabs), and released by digestion with HindIII. To obtain a labelled probe at the 5' end of the antisense strand, the vector was digested with HindIII first, then BglII. The footprint assay was performed as described previously²². The GA cleavage ladder was carried out according to the Maxam–Gilbert sequencing procedure. Recombinant his-tagged Miz-1 was purified from BL21 cells transformed with a pTrcHis2B vector encoding the Miz-1 cDNA²².

Immunofluorescence

HCT116 or HaCaT cells were incubated in the presence or absence of daunorubicin or TGF- β , respectively, for the specified times, fixed with paraformaldehyde, and subjected to immunofluorescence analysis with Myc or p21 antibodies.

Chromatin immunoprecipitation

Cells were grown to 70% confluence, left untreated or treated with daunorubicin for 15 h, and subsequently crosslinked with 1% formaldehyde at room temperature for 10 min. Chromatin immunoprecipitations were performed essentially as previously described³⁰.

Flow cytometric cell cycle analysis

Cells were fixed in 66% methanol, treated with RNase A (100 mg ml⁻¹) and propidium iodide (5 mg ml⁻¹), and analysed using a FACScan apparatus (Becton Dickinson).

Cdk2 kinase activity

Cells were lysed in TNE buffer and 100 µg of cell extract was immunoprecipitated and analysed as described in ref. 26.

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Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation

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Engagement of the NKG2D receptor by tumour-associated ligands may promote tumour rejection by stimulating innate and adaptive lymphocyte responses^{1–5}. In humans, NKG2D is expressed on most natural killer cells, γδ T cells and CD8αβ T cells¹. Ligands of NKG2D include the major histocompatibility complex class I homologues MICA and MICB, which function as signals of cellular stress^{6,7}. These molecules are absent from most cells and tissues but can be induced by viral and bacterial infections and are frequently expressed in epithelial tumours^{8–11}. MIC engagement of NKG2D triggers natural killer cells and costimulates antigen-specific effector T cells^{1,10}. Here we show that binding of MIC induces endocytosis and degradation of NKG2D. Expression of NKG2D is reduced markedly on large numbers of tumour-infiltrating and matched peripheral blood T cells from individuals with cancer. This systemic deficiency is associated with circulating tumour-derived soluble MICA, causing the downregulation of NKG2D and in turn severe impairment of the responsiveness of tumour-antigen-specific effector T cells. This mode of T-cell silencing may promote tumour immune evasion and, by inference, compromise host resistance to infections.

Ectopic expression of NKG2D ligands causes rejection of trans-fected tumour cells by natural killer (NK) cells and primed cytotoxic T cells in syngeneic mice^{2,3}. Immunity is induced against subsequent challenges with tumour cells that lack NKG2D ligands³. This evidence reinforces the potential significance of human MIC–NKG2D ligand–receptor signalling in immune responses against tumours. But the presence of MIC on many progressing tumours, including breast, lung, gastric, renal, colon, ovarian and prostate carcinomas and melanomas⁸, suggests that MIC or NKG2D might be functionally impaired, thereby promoting immune evasion.

We examined NKG2D expression on CD8⁺ T cells among tumour infiltrating lymphocytes (TILs) extracted from 39 epithelial tumours that were either positive or negative for MIC by mono-clonal antibody staining of cell suspensions and flow cytometry. In all 13 MIC-negative tumour specimens examined, the surface amounts of NKG2D were unaltered as compared to normal control peripheral blood mononuclear cells (PBMCs; ref. 1, Fig. 1a and data not shown). By contrast, the mean fluorescence intensities of NKG2D on CD8⁺ T cells from all 26 MIC-positive tumours tested were reduced by about 60–70% (Fig. 1b). This observation was not dependent on the proportions of MIC-positive tumour cells, which ranged from 40 to 100% (ref. 8 and data not shown). Expression of NKG2D was similarly reduced on T cells among matched PBMCs (Fig. 1b). This deficiency was not associated with T-cell subsets defined by differentiation or activation markers CD28, CCR7, CD45 isoforms and CD69. Substantially reduced expression of NKG2D was also observed on CD56⁺ NK cells and γδ T cells (data not shown). These results showed that a systemic impairment of NKG2D is linked to the presence of tumour-associated MIC.

We examined the apparent effect of MIC on NKG2D expression by culturing normal PBMCs together with C1R-MICA (or C1R-MICB) B-cell transfectants, untransfected controls, and freshly isolated MIC-positive melanoma cells. We used conditions favouring conjugate formation, and examined the cells by three-colour

staining of dissociated cells with monoclonal antibodies against CD3, CD8 and NKG2D, and flow cytometry. After 6 h, the average amounts of surface NKG2D began to decline and reached a maximum five- to eightfold reduction between 24 and 48 h in the presence (Fig. 2a; data not shown) but not in the absence (Fig. 2b) of MIC. Similar results were obtained with melanoma-antigen-specific HLA-A2-restricted T-cell clones after co-culture with MART-1- or gp100-peptide-pulsed C1R-A2-MICA transfectants, but not with identically treated C1R-A2 cells (data not shown). These results indicate that MIC-induced downregulation of NKG2D is independent of T-cell antigen receptor (TCR) stimulation.

As many receptors undergo ligand-induced endocytosis followed by recycling or degradation^{12–14}, we explored the fate of NKG2D by measuring its surface and total cellular amounts by using random CD8⁺ $\alpha\beta$ T-cell clones exposed for 12 h to C1R-MICA cells and flow cytometry. As with the surface protein, the mean fluorescence intensity reflecting the total NKG2D content of permeabilized cells was strongly reduced as compared to control T cells exposed to C1R cells (Fig. 2c). But treating T cells with bafilomycin A1 or chloroquine, which are inhibitors of lysosomal degradation, prevented this decrease in total cellular NKG2D (Fig. 2c; data not shown). Inhibition of protein synthesis with cycloheximide had no effect. Thus, at least a large proportion of endocytosed NKG2D was not recycled but degraded.

These results offered an explanation for the deficiency of NKG2D

among TILs, which directly contact MIC-positive tumour cells, but not PBMCs. We therefore considered that tumour-associated MIC might be shed into the circulation, possibly as a result of tumour cell death, secretion of exosomes or proteolysis, or a combination thereof. By a sandwich enzyme-linked immunosorbent assay (ELISA) using noncompeting solid-phase capture and biotinylated detection monoclonal antibodies 6G6 (specific for the $\alpha 3$ domain of MICA and MICB)⁷ and 2C10 (specific for the MICA $\alpha 1\alpha 2$ domains)⁶ respectively, we detected a minimum of 1.5 ng ml⁻¹ purified recombinant soluble MICA (rsMICA) after reaction with streptavidin-conjugated horse radish peroxidase (HRP) and tetramethyl-benzidine substrate (Fig. 3).

We obtained positive results, indicating maximum amounts of about 25–50 ng ml⁻¹ soluble MICA (sMICA), with 7 of 14 blood serum samples from individuals with tumours and a NKG2D^{low} phenotype (2/4 breast, 3/3 lung, 0/2 ovarian and 0/1 colon tumours, and 2/4 melanomas). This incomplete correlation might be due to technical limitations including the sensitivity of the ELISA and its lack of detection of sMICB. All six samples from individuals with normal NKG2D expression gave negative results (Fig. 3). sMICA was also detected in cleared supernatants of colon (HCT-116), prostate (PC3), ovarian (HTB-79) and melanoma (375) tumour cell lines grown to high confluency, but not in supernatants of transfected B cell lines (data not shown). Thus, the shedding of MIC was a property of epithelial tumour cells. Incubation of random CD8⁺ $\alpha\beta$ T-cell clones for 24 h with rsMICA resulted in fivefold reductions in the mean surface quantities of NKG2D (Fig. 4a). T-cell-bound rsMICA was detected in the early incubation phase

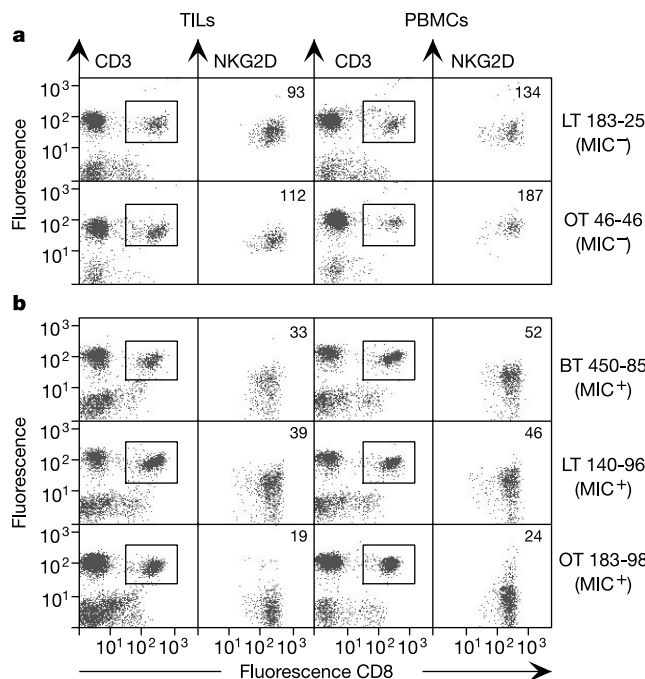


Figure 1 Reduced expression of NKG2D on CD8⁺ $\alpha\beta$ T cells among TILs and PBMCs from individuals with MIC-positive tumours. **a**, Three-colour flow cytometry analysis with gating on CD3⁺ (y axis) CD8⁺ (x axis) T cells (dot plot columns 1 and 3) shows that the surface amounts of NKG2D (y axis; dot plot columns 2 and 4) are normal when tumours lack expression of MIC (see ref. 1 for comparison with T cells from healthy individuals). Dot plots shown are representative of 13 matched MIC-negative tumour and PBMCs samples (2 breast, 6 lung, 1 ovarian and 2 colon tumours, and 2 melanomas). **b**, Reduced expression of NKG2D among CD3⁺CD8⁺ $\alpha\beta$ T cells from TILs and PBMCs associated with MIC-positive tumours (see ref. 8 for representative tumour cell expression profiles of MIC). Dot plots shown are representative of six breast, five lung, four ovarian and three colon carcinomas, and eight melanomas. The fluorescent labels are described in Methods. Numbers in the top right corners represent the NKG2D mean fluorescence intensities derived from histograms. Tumour specimens are given on the right: BT, breast tumour; LT, lung tumour; OT, ovarian tumour.

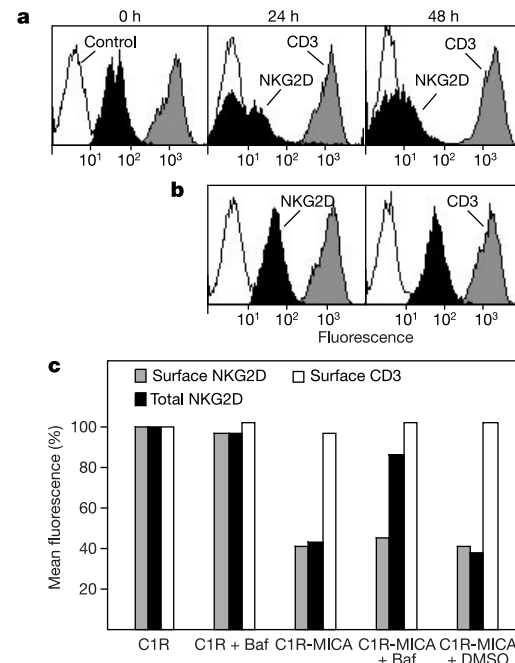


Figure 2 MIC-induced downregulation and degradation of NKG2D. **a**, CD3⁺CD8⁺ T cells among normal PBMCs show five- to eightfold reductions in the mean fluorescence intensity staining of NKG2D after co-culture with C1R-MICA transfectants. **b**, No effect is observed with untransfected C1R cells. Open profiles, IgG1 isotype control staining; shaded profiles, CD3 staining; filled profiles, NKG2D staining. **c**, Endocytosis and degradation of NKG2D. After co-culture of CD8⁺ $\alpha\beta$ T-cell clones derived from peripheral blood with C1R-MICA transfectants but not with C1R cells, both cell-surface and total cellular NKG2D are substantially reduced. Treating T cells with bafilomycin A1 prevents the decrease in total cellular NKG2D. The solvent control is DMSO. Surface CD3 remains unchanged. The mean fluorescence intensities obtained after exposure to negative control C1R cells are arbitrarily set as 100%. Data are representative of five independent experiments.

but not after 24 h, presumably because of degradation. Thus, rsMICA did not interfere with binding of the monoclonal antibody against NKG2D (Fig. 4a).

These results suggested that tumour-derived sMICA and presumably sMICB in peripheral blood of individuals with cancer causes the downregulation of NKG2D. This hypothesis was confirmed by experiments showing that sera from individuals with MIC-positive tumours, but not from negative controls, down-regulated NKG2D expression on CD8⁺ T cells among normal PBMCs even when sMICA was undetectable by ELISA, and that this activity was neutralized in the presence of a monoclonal antibody specific for MIC (Fig. 5). At least in part, this capacity of sMIC may be explained by its high binding affinity for NKG2D^{15,16}. With peripheral blood T cells, the low expression of NKG2D was reversed after 7–8 d of culture (data not shown), or more rapidly after 24 h in the presence of a monoclonal antibody against CD3. But the effect of TCR complex stimulation was suppressed by the addition of rsMICA (Fig. 4b).

Ligand engagement of NKG2D augments cytolytic responses under conditions of suboptimal T-cell activation and costimulates cytokine production and T-cell proliferation^{9,10}. Signalling is mediated by DAP10, an adaptor protein that is associated with NKG2D¹⁷. To assess the functional impairment of NKG2D^{low} T cells, we tested MART-1-specific T-cell clones¹⁸ with rsMICA-induced low expression of NKG2D (Fig. 6a) against MIC-positive melanoma target cells pulsed with titred concentrations of the antigenic M27 peptide. Within a range of suboptimal peptide concentrations, the cytolytic responses of the NKG2D^{low} T cells were substantially lower than those of untreated NKG2D^{high} T cells (Fig. 6b). We also tested the responses of freshly isolated TILs (HLA-A2⁺) from MIC-negative and MIC-positive melanomas to specific MHC-peptide antigen and MIC stimulation. CD8⁺ T cells purified by negative selection were stimulated for short periods with irradiated C1R-A2-MICA transfectants pulsed with M27 peptide. Antigen-specific T cells were labelled with HLA-A2-M27 tetramer¹⁸ and stained for NKG2D. Fixed and permeabilized cells were stained for interferon- γ (IFN- γ), which could accumulate intracellularly owing to experimental inhibition of the secretory pathway. Analysis by flow cytometry showed induction of IFN- γ in a substantial proportion of NKG2D^{high} (Fig. 6d) but not NKG2D^{low} (Fig. 6c) T cells. Thus, T-cell stimulation by NKG2D was profoundly impaired.

Together, our results indicate that tumour shedding of MIC systemically impairs the responses of CD8 $\alpha\beta$ T cells and presumably NK cells, thus compromising the immunological competence

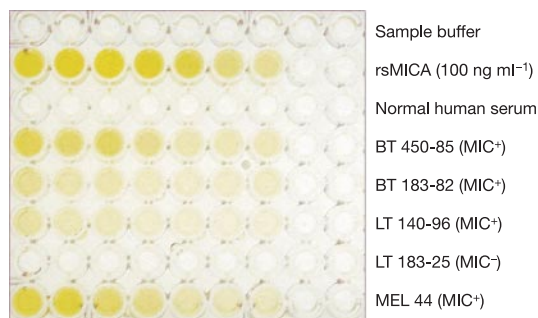


Figure 3 Detection of sMICA by ELISA in serum samples from individuals with MIC-positive tumours and reduced expression of NKG2D. Horizontal wells from left to right are serial 1:2 dilutions of sample buffer, rsMICA (100 ng ml⁻¹), normal human serum, four serum samples from individuals with MIC-positive tumours (BT 450-85, BT 183-82, LT 140-96 and MEL 44) and a sample from an individual with a MIC-negative tumour (LT 183-25). Results are representative of seven MIC-positive samples (two breast and three lung tumours, and two melanomas) and six MIC-positive samples (one breast and five lung tumours). BT, breast tumour; LT, lung tumour; MEL, melanoma.

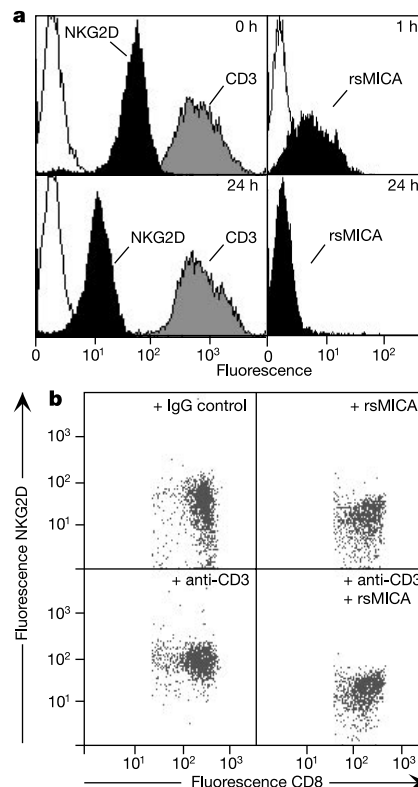


Figure 4 Downregulation of NKG2D by rsMICA. **a**, Left, incubation of rsMICA (100 ng ml⁻¹) with CD8⁺ $\alpha\beta$ T-cell clones derived from peripheral blood results in reduced surface amounts of NKG2D. A similar but less pronounced effect is induced by much lower concentrations of rsMICA (10 ng ml⁻¹; not shown). Right, cell-associated rsMICA is detected after 1 h but not after 24 h of incubation. Open profiles, IgG1 isotype control staining. Data are representative of experiments with five different T-cell clones. **b**, Melanoma-derived TILs gated on CD3⁺CD8⁺ T cells show further decreases in expression of NKG2D after incubation with rsMICA (top). NKG2D was induced by a monoclonal antibody against CD3 but not in the additional presence of rsMICA (bottom).

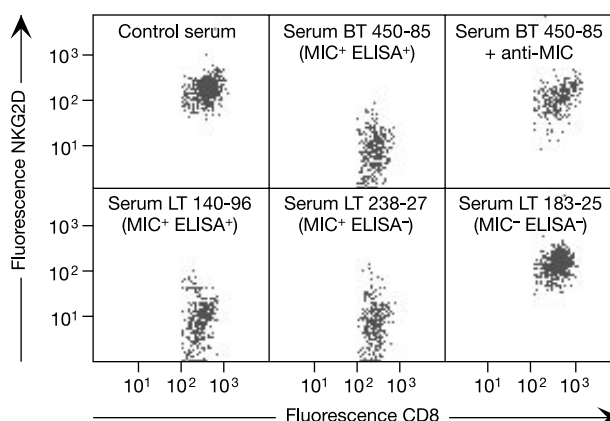


Figure 5 Downregulation of NKG2D by sera from individuals with MIC-positive tumours. The fluorescence intensity of NKG2D measured by staining with monoclonal antibody 1D11 (ref. 1) and FITC-conjugated goat antibody against mouse IgG on CD8⁺ T cells among normal PBMCs is markedly diminished after 24 h of incubation in 1:5 dilutions of sera from individuals with MIC-positive breast or lung tumours (BT 450-85, LT 140-96 and LT 238-27). This activity is neutralized in the presence of 6D4 monoclonal antibody against MIC⁷ (top right plot) and is not observed with normal control serum or with serum from an individual with a MIC-negative lung tumour (LT 183-25). Note that NKG2D is downregulated by serum from an individual with a MIC-positive tumour (LT 238-27) in which sMICA is undetectable by ELISA.

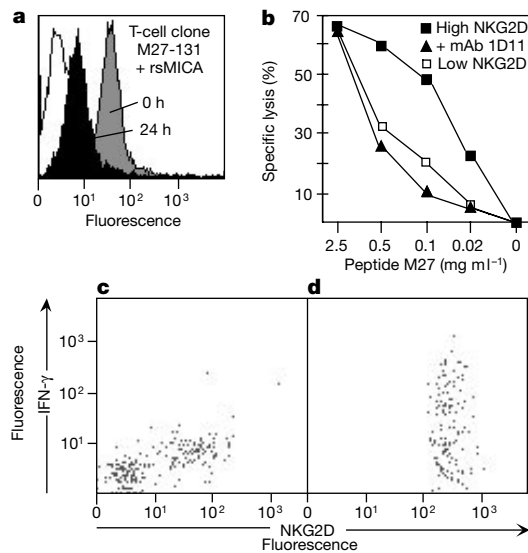


Figure 6 Functional impairment of NKG2D^{low} T cells. **a**, Incubation with rsMICA reduces NKG2D expression on the HLA-A2-restricted and MART-1-specific M27-131 T cells¹⁸. **b**, Under conditions of suboptimal antigen stimulation of TCR, NKG2D^{low} M27-131 T cells respond poorly against melanoma 375 cells pulsed with titred concentrations of M27 peptide. Untreated NKG2D^{high} M27-131 T cells show augmented cytolytic responses¹⁰. The 1D11 monoclonal antibody¹ is specific for NKG2D. **c**, NKG2D^{low} CD8⁺ αβ T cells isolated from TILs from a MIC-positive melanoma and labelled with HLA-A2-M27 tetramer show no or little induction of IFN-γ after stimulation with C1R-A2-MICA transfectants pulsed with M27 peptide. **d**, A substantial proportion of identically treated NKG2D^{high} M27-specific T cells from a MIC-negative melanoma produce a strong IFN-γ response.

of individuals with cancer. Although expression of MIC by nascent tumours might, to some extent, be effective in mobilizing responses from effector T cells and NK cells, its shedding at progressive stages of tumour growth probably promotes immune evasion. Notably, the factors that determine the tumour-associated expression of MIC and the shedding mechanism remain to be defined. But monitoring the presence of sMICA in tissue or peripheral blood samples might be applicable to the prognosis or diagnosis of cancer, just as the MIC–NKG2D system might offer possibilities for therapy. □

Methods

Tumour specimens and PBMCs

Specimens of 8 breast, 11 lung, 5 ovarian and 5 colon carcinomas and of 10 melanomas from diagnostic or therapeutic resections and matched peripheral blood samples were a gift from H. Secrist and D. Byrd and R. Yeung. Tumours were diagnosed by histopathological criteria. We obtained normal PBMCs from random healthy volunteers. These activities were approved by local institutional review boards and all subjects gave written informed consent. We prepared TILs and tumour cell suspensions as described⁸. PBMCs were purified by Ficoll/Hypaque density centrifugation.

Antibodies and flow cytometry

We stained suspensions of tumour cells with monoclonal antibody 6D4 (against MICA and MICB)⁷ and phycoerythrin (PE)-conjugated goat antibodies against mouse F(ab')₂ (Southern Biotechnologies), and analysed them using a FACScan (Beckton Dickinson) flow cytometer. TILs and PBMCs were examined by three-colour flow cytometry using various combinations of antibodies against CD3, CD4, CD8, CD56, TCRγδ, CD28, CD69, CCR7, CD45RO and CD45RA conjugated to PE, fluorescein isothiocyanate (FITC), PerCP or allophycocyanin (BD/PharMingen). We detected binding of the 1D11 monoclonal antibody against NKG2D (ref. 1) to TILs, PBMCs and T-cell clones with PE- or FITC-conjugated goat antibodies against mouse F(ab')₂.

Modulation of NKG2D

Freshly isolated PBMCs were co-cultured with irradiated C1R-MICA transfectants, C1R cells or freshly isolated MIC-positive melanoma cells (at 1:2 ratios) after a brief low-speed centrifugation in round-bottom microtitre plates. After various durations, the cells were washed in PBS plus 0.5% EDTA and stained for expression of CD3, CD8 and NKG2D. We gated transfectants, C1R and tumour cells using forward- and side-scatter parameters. Similarly, HLA-A2-restricted CD8 αβ T-cell clones specific for MART-1 (M27 peptide AAGIGILTIV)¹⁸ or gp100 (G154 peptide KTWGQYVQV)¹⁸ were cultured together with

C1R-A2-MICA or C1R-A2 transfectants pulsed with optimal concentrations (2.5 μg ml⁻¹) of the appropriate peptide.

We showed ligand-induced endocytosis and degradation of NKG2D with random CD8⁺ αβ T-cell clones that were established as described¹. We cultured T cells for 12 h with C1R-MICA or C1R control cells, washed them and fixed them with paraformaldehyde. Half of the cells were stained for surface CD3, CD8 and NKG2D; the other half were stained after permeabilization in 0.1% saponin. Continuous exposure of T cells to bafilomycin A1 (Sigma; 0.5 μM) or chloroquine (33 μM) was used to inhibit lysosomal degradation. We used dimethyl sulphoxide (DMSO; 1%) as the solvent control¹⁴.

Downregulation of NKG2D by rsMICA was shown by incubating random CD8 αβ T-cell clones with rsMICA (10 or 100 ng ml⁻¹) for various durations before flow cytometry analysis of surface NKG2D. Surface-associated rsMICA was monitored with monoclonal antibody 2C10 (ref. 6). NKG2D^{low} TILs extracted from a MIC-positive melanoma were used for induction of NKG2D by a plate-bound antibody against CD3 (50 ng ml⁻¹) after 24 h of incubation. Induction was prevented by rsMICA (100 ng ml⁻¹). Downregulation of NKG2D by sera from individuals with MIC-positive tumours was shown by incubation of freshly isolated normal PBMCs with 1:5 dilutions of sera (BT 450-85, LT 140-96 and LT 238-27) for 24 h before flow cytometry analysis. Normal serum and serum from an individual with a MIC-negative lung tumour (LT 183-25) were used as negative controls.

ELISA and rsMICA

High-binding EIA/RIA polystyrene plates (Corning) were coated with capture 6G6 monoclonal antibody (10 μg ml⁻¹ PBS, 50 μl per well) for 10 h at 4 °C, washed with PBS plus 0.1% Tween 20, blocked overnight at 4 °C with blocking buffer (215 μl of 20 mM Tris-OH, pH 7.5, 150 mM NaCl, 5% EIA grade bovine serum albumin (BSA) and 0.05% Tween 20), and washed with PBS plus 0.1% Tween 20. Serial 1:2 dilutions in sample buffer (blocking buffer containing 0.1% BSA and 0.1% Tween 20) of rsMICA and patient and control serum samples were plated at 50 μl per well, incubated for 2 h at room temperature on a shaker and washed five times in PBS plus 0.1% Tween 20. After incubation under the same conditions with 50 μl of biotinylated 2C10 detection monoclonal antibody (1.2 μg ml⁻¹ sample buffer), washed plates were incubated for 30 min at room temperature with 50 μl of streptavidin–HRP (Vector Laboratories), washed and reacted for 7–8 min with tetramethyl-benzidine (Kirkegaard and Perry Laboratories). We stopped the reactions with 1 M phosphoric acid (100 μl per well) and scored them at a wavelength of 450 nm using a Veramax microplate reader (Molecular Devices).

rsMICA was expressed as a secreted protein in High-Five insect cells (Invitrogen) using the BAC-TO-BAC Baculovirus expression system (GibcoBRL)¹⁵. The extracellular sequences of MICA corresponding to the signal peptide and the α1α2α3 domains were amplified from template complementary DNA, using oligonucleotide primers and PCR, and purified. In subsequent rounds of PCR and amplicon purification, the truncated MICA sequence was fused to a 15-residue biotinylation recognition sequence¹⁹, which was not used in this study, using the primers 5'-TCTGGATCCATGGGGCTGGGCCCGGTC-3' (MICA 5' end) and 5'-ACGATGAATCCACACCATTTCTGTGCATCAGAATATGATG CAGGCTTCCTTCCAGAGGGGACAGGGGTAG-3' (biotinylation recognition sequence/glycine-serine linker/MICA 3' end overlap), and to sequences for a hexahistidine tract and the eight-amino-acid Flag tag (DYKDDDDK) M2 antibody epitope²⁰. The amplicon was flanked by restriction sites for insertion into pFASTBAC1 (GibcoBRL). We transfected the sequenced construct into Sf9 cells for production of high-titre recombinant Baculovirus, which was used to infect High-Five cells. Media were collected from cell cultures grown in spinner flasks 4–5 d after infection, cleared, filtered and applied to a Ni²⁺-charged Chelating Sepharose Fast Flow resin column (Pharmacia). Recombinant protein was eluted with imidazol in PIPES, NaCl, Na₃ buffer, further purified by Superdex S75 gel filtration chromatography (Pharmacia)¹⁵, and concentrated using a BIOMAX-100K (Millipore) ultrafiltration device. rsMICA was a monomeric protein as judged by SDS-PAGE.

T-cell function assays

We cultured HLA-A2-restricted and MART-1-specific M27-131 T cells¹⁸ for 24 h with 100 ng ml⁻¹ rsMICA to induce low expression of NKG2D, washed them in culture media and then tested them in standard ⁵¹Cr-release assays against MIC-positive melanoma 375 cells (American Type Culture Collection) that had been pulsed with titred concentrations of M27 peptide. Induction of intracellular IFN-γ was carried-out with CD8⁺ αβ T cells obtained by negative selection with RosetteSep (StemCell Technologies) from TILs isolated from MIC-positive and MIC-negative melanomas. We stimulated T cells for 4 h with equal numbers of C1R-A2-MICA transfectants pulsed with M27 peptide in the presence of monensin (GolgiStop, PharMingen) as described¹⁰. M27-specific T cells were identified with PE-conjugated HLA-A2-M27 tetramer¹⁸ and stained for NKG2D. After being fixed and permeabilized by a Cytotfix/Cytoperm Plus kit (PharMingen), T cells were stained for intracellular IFN-γ with a specific allophycocyanin-conjugated monoclonal antibody (PharMingen) and analysed by flow cytometry¹⁰.

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A transcription-factor-binding surface of coactivator p300 is required for haematopoiesis

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The coactivators CBP (Cre-element binding protein (CREB)-binding protein) and its paralogue p300 are thought to supply adaptor molecule and protein acetyltransferase functions to many transcription factors that regulate gene expression¹. Normal development requires CBP and p300, and mutations in these genes are found in haematopoietic and epithelial tumours^{2–6}. It is unclear, however, which functions of CBP and p300 are essential *in vivo*. Here we show that the protein-binding KIX domains of CBP and p300 have nonredundant functions in mice. In mice homozygous for point mutations in the KIX domain of p300 designed to disrupt the binding surface for the transcription factors c-Myb and CREB^{7–9}, multilineage defects occur in haematopoiesis, including anaemia, B-cell deficiency, thymic hypoplasia, megakaryocytosis and thrombocytosis. By contrast, age-matched mice homozygous for identical mutations in the KIX domain of CBP are essentially normal. There is a synergistic

genetic interaction between mutations in c-Myb and mutations in the KIX domain of p300, which suggests that the binding of c-Myb to this domain of p300 is crucial for the development and function of megakaryocytes. Thus, conserved domains in two highly related coactivators have contrasting roles in haematopoiesis.

The KIX domain is one of several domains in CBP (also known as Crebbp) and p300 that bind transcriptional regulators (Fig. 1a); it is highly conserved in evolution, with 90% identity in human CBP and p300 (Fig. 1b). The three-dimensional structure of the CBP KIX domain in a complex containing a portion of the CREB activation domain has been determined (ref. 9 and Fig. 1c). Specific hydrophobic residues on the surface of the KIX domain are important for binding the α -helical activation domains of CREB and c-Myb^{7,8}.

We tested the role of the KIX domain transcription factor-binding surface *in vivo* by mutating three highly conserved residues that lie on one face of the α 3 helix in the KIX domain of CBP (Fig. 1b, c): Tyr 650 forms part of the hydrophobic surface that makes crucial contacts with CREB; the small side chain of Ala 654 allows the close packing of CREB; and Tyr 658 forms hydrophobic interactions with CREB and forms hydrogen bonds with phosphorylated Ser 133 of CREB⁹. Thus, we thought that replacing Tyr 650 and Tyr 658 with alanines should disrupt bonding with CREB, and replacing the Ala 654 methyl group with a bulkier glutamine side chain should sterically hinder CREB binding. Accordingly, the individual mutations of Tyr650Ala, Ala654Gln, and Tyr658Ala each diminish CREB and c-Myb binding to KIX (refs 7, 8, and data not shown). Structure prediction analyses using AGADIR and PSIPRED predicted that KIX secondary structure would not be affected by a Tyr650Ala, Ala654Gln, Tyr658Ala triple mutation (refs 10, 11, and data not shown).

We introduced this triple mutation into the CBP and p300 loci of embryonic stem (ES) cells by homologous recombination (Fig. 1d, e). For p300, the corresponding residues, Tyr 630, Ala 634 and Tyr 638, were mutated. Correctly targeted ES cells were used to produce mice carrying mutant alleles of the KIX domain of CBP (designated *CBP*^{KIX}) or p300 (designated *p300*^{KIX}; Fig. 1d, e). *CBP*^{KIX/KIX} and *p300*^{KIX/KIX} homozygous mice were viable, although some died of unknown causes before they were 3 weeks old (mostly *p300*^{KIX/KIX} mice; see Supplementary Information). Surviving 4-week-old *p300*^{KIX/KIX} mice averaged about 50–70% of the size of wild-type or heterozygous littermates, and *CBP*^{KIX/KIX} mice also tended to be smaller than their littermate controls. Analysis of the surviving *CBP*^{KIX/KIX} age-matched mice was unrevealing apart from a slight reduction in thymocyte numbers (Supplementary Information). By contrast, *p300*^{KIX/KIX} mice had a marked reduction in thymocyte numbers (~5% of wild type) and severe anaemia (haematocrit 20.2 ± 5.3 versus $42.3 \pm 1.4\%$ for wild type) and thrombocytosis ($(5.0 \pm 1.1) \times 10^9$ versus $(1.2 \pm 0.2) \times 10^9$ platelets per ml for wild type). Numbers of neutrophils were generally in the normal range (data not shown). The blood from *p300*^{KIX/KIX} mice showed increased variation in erythrocyte size (compare Fig. 2b with Fig. 2a, c) and the presence of megathrombocytes (Fig. 2b, broken arrow). *CBP*^{+/KIX} and *p300*^{+/KIX} mice were essentially normal (except for a small increase in platelets in *p300*^{+/KIX} mice), which showed that the mutations were not overtly dominant (Supplementary Information).

The bone marrow of *p300*^{KIX/KIX} mice showed megakaryocytic hyperplasia with a corresponding decrease in other bone marrow elements (compare Fig. 2d with Fig. 2e). Megakaryocytosis was evident in the spleens of these mice (compare Fig. 2g with Fig. 2f, h) and was indicated further by widespread staining for acetylcholinesterase enzyme (compare Fig. 2j with Fig. 2i) and factor VIII (data not shown). Megakaryocytosis in *p300*^{KIX/KIX} mice was also indicated by flow cytometric analysis of bone marrow and spleen cells, which contained an overabundance of CD41-positive cells as compared with *CBP*^{KIX/KIX} and wild-type mice (Fig. 2k). Serum

concentrations of thrombopoietin (Tpo), which stimulates megakaryocytopoiesis, were reduced about fivefold in $p300^{KIX/KIX}$ mice (probably owing to Tpo internalization and degradation by excess platelets), which indicated that overproduction of Tpo was not the cause of the megakaryocytosis (Supplementary Information). We assessed the development of $p300^{KIX/KIX}$ megakaryocytes by quantifying the DNA content in CD42d-positive bone marrow cells (Fig. 2l). $p300^{KIX/KIX}$ mice had more immature megakaryocytes with a modal ploidy of $8n$, as compared with $16n$ in control mice.

Thymuses from $p300^{KIX/KIX}$ mice had an apparently normal distribution of single- and double-positive CD4 and CD8 thymocytes (Fig. 2n). Despite the decreased absolute number of $p300^{KIX/KIX}$ thymocytes, peripheral single-positive CD4 and CD8 T-cell populations seemed to be in the normal range (data not shown). By contrast, there was a deficit of B220-positive, immunoglobulin- μ (IgM)-positive, immunoglobulin- δ (IgD)-positive mature B cells in $p300^{KIX/KIX}$ mice (Fig. 2m).

To determine whether the $p300^{KIX/KIX}$ phenotype was intrinsic to the bone marrow, we transplanted $p300^{KIX/KIX}$ bone marrow cells into wild-type mice that had been lethally irradiated. Mice that received $p300^{KIX/KIX}$ bone marrow cells developed a phenotype comparable to that of $p300^{KIX/KIX}$ mice, with severe anaemia, thrombocytosis and B-cell deficiency, indicating that $p300^{KIX/KIX}$ bone marrow cells were sufficient to confer the disease (Supplementary Information, and data not shown). A mix of $p300^{KIX/KIX}$ bone marrow cells with equal numbers of wild-type cells conferred a milder anaemia with thrombocytosis (Supplementary Information).

The stability and expression of CBP and p300 proteins were unaffected by mutations in the KIX domain, as judged by western blot analysis of nuclear extracts from the brains of $CBP^{KIX/KIX}$ and

$p300^{KIX/KIX}$ mice (Fig. 3a). Histone acetyltransferase (HAT) activities of immunoprecipitated mutant CBP and p300 were also indistinguishable from those of wild-type mice (Fig. 3b). By contrast, several independently derived $CBP^{KIX/KIX}$ and $p300^{KIX/KIX}$ primary mouse embryonic fibroblast (MEF) lines were compromised in their ability to support CREB and c-Myb activity in transient transfection assays by about two- to fourfold as compared with wild-type MEFs. This was true for both endogenous CREB and ectopically expressed c-Myb and the Gal4 DNA-binding-domain fused to CREB or c-Myb (Fig. 3c–e). Notably, Gal4 fusions of activators that depend on other domains of CBP and p300 or other coactivators (Ets-1 and VP16) were unaffected in $p300^{KIX/KIX}$ MEFs (refs 12–14 and Fig. 3d, e). Expression of wild-type CBP or p300, but not mutant CBP, could restore Gal4–CREB activity in $CBP^{KIX/KIX}$ MEFs (Fig. 3f, g). Cyclic-AMP-dependent induction of the endogenous CREB target genes encoding c-Fos, JunB and the ICER form of CREM were not measurably different in mutant and wild-type MEFs, which suggests that the KIX domain is not limiting for these genes in MEFs (data not shown). Together, these data indicate that a 50% reduction in the expression of wild-type KIX domains results in the specific attenuation of CREB and c-Myb activities in certain promoter contexts.

We compared CBP and p300 proteins in MEFs, megakaryocytes, thymus and relatively unaffected tissues from wild-type mice. Western blot analysis of nuclear extracts made from highly enriched megakaryocytes showed increased concentrations of p300 relative to CBP when compared with brain, heart and MEFs (Fig. 4a). The thymus also had a higher p300/CBP protein ratio (data not shown). We verified the increased ratio of p300 to CBP in megakaryocytes by analysing mRNA levels in megakaryocyte and brain by quantitative polymerase chain reaction with reverse transcription (RT–PCR). $p300$ mRNA was about six times more abundant than CBP mRNA in

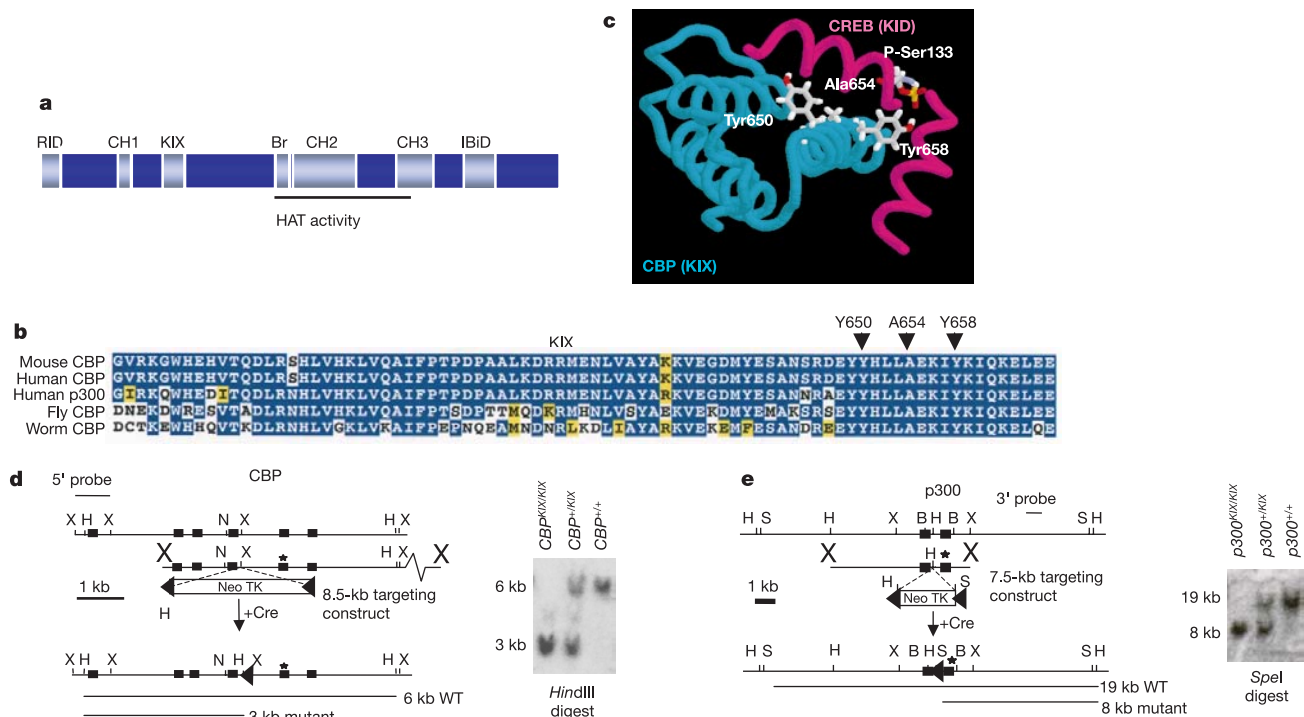


Figure 1 Mouse KIX domain mutations. **a**, Domains in CBP and p300: nuclear receptor interaction domain (RID), cysteine/histidine-rich domains (CH1, CH2, CH3), KIX bromodomain (Br), IRF3-binding domain (IBiD) and HAT. **b**, KIX domains from mouse, human, *Drosophila melanogaster* and *Caenorhabditis elegans*. Conserved residues are indicated. Produced with Boxshade. **c**, Structure of the KIX domain from CBP (green) and the KID domain from CREB (magenta) with the side chains of Tyr 650, Ala 654, Tyr 658

and the phosphoserine 133 (P-Ser133) of CREB highlighted. Produced using Protein Explorer and data from the Protein Data Bank. **d**, **e**, Targeting strategies for CBP (**d**) and p300 (**e**), and Southern blots of mouse tail DNA. Star indicates a mutated exon, filled boxes indicate probes and exons and arrowhead indicates *loxP* site. X, *Xba*; H, *HindIII*; B, *BamHI*; N, *NheI*; S, *SpeI*.

megakaryocytes (t -test, $P < 0.01$; Fig. 4b). Protein binding assays using glutathione S -transferase (GST)–Myb and GST–CREB did not identify tissue-specific differences in the binding of CBP and p300 from nuclear extracts, which suggests that the KIX domains of CBP and p300 are functionally equivalent when isolated from different sources (data not shown). Thus, cells with an increased p300/CBP ratio conceivably might be more susceptible to mutations in the KIX domain of p300.

Because c -Myb homozygous null mice have a deficiency in haematopoiesis^{15,16}, we investigated whether defects in the $p300^{KIX/KIX}$ mice were caused by an attenuation of c -Myb activity. We examined mice with a single c -Myb null allele, either alone or in combination with a single $p300^{KIX}$ gene. For c -Myb^{+/-} mice we reasoned that if mutations in KIX lead to a reduction in c -Myb function, then decreased concentrations of c -Myb might result in a $p300^{KIX/KIX}$ -like phenotype. Similarly, mice heterozygous for

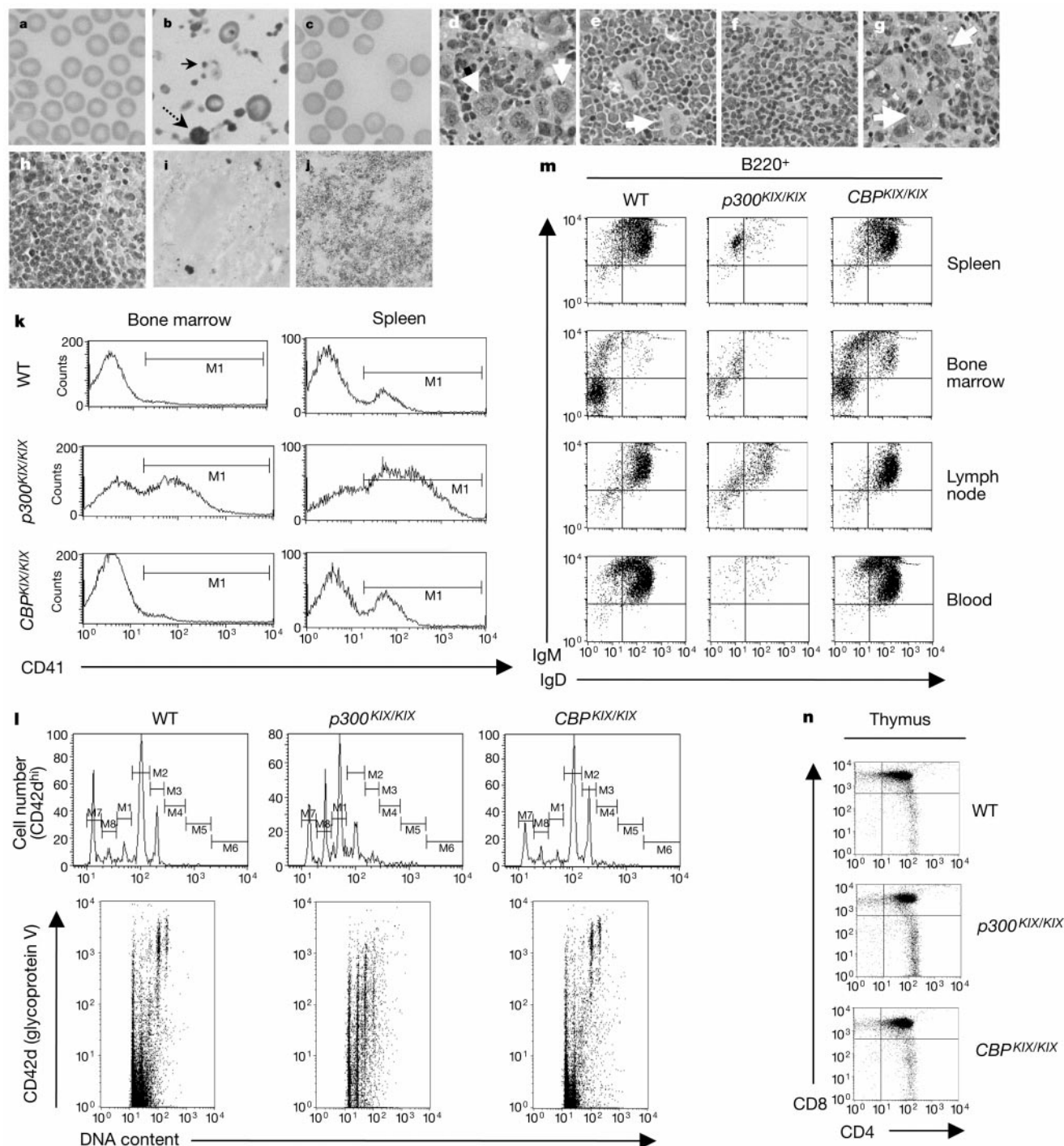


Figure 2 Haematopoietic defects in $p300^{KIX/KIX}$ mice. **a–j**, Blood (**a–c**), bone marrow (**d, e**), spleen (**f–j**) from wild-type (**a, f, i**), $p300^{KIX/KIX}$ (**b, d, g, j**) and $CBP^{KIX/KIX}$ (**c, e, h**) mice; examples are shown of giant (broken arrow) and normal (small arrow) platelets and megakaryocytes (large arrows). Acetylcholinesterase-positive megakaryocytes in spleen are shown at low magnification (**i, j**). **k**, CD41-positive megakaryocytes in $p300^{KIX/KIX}$

bone marrow and spleen. **l**, $p300^{KIX/KIX}$ megakaryocytes have low modal ploidy ($8n$). The peaks in the histogram (top) correspond to $2n$ to $32n$ cells (left to right). **m**, B-cell deficiency in $p300^{KIX/KIX}$ mice. **n**, Normal population distribution of double-positive CD4 CD8 thymocytes in $p300^{KIX/KIX}$ mice.

mutations in both c-Myb and the KIX domain of p300 might show a synergistic phenotype if the interaction between c-Myb and the KIX domain of p300 is limiting for c-Myb-dependent transcription and haematopoietic development^{17,18}.

Two- to three-week-old *c-Myb*^{+/-}*p300*^{+KIX} double heterozygous mice had a large increase in platelets ($(3.05 \pm 0.47) \times 10^9$ versus $(1.22 \pm 0.22) \times 10^9$ platelets per ml for wild type; *t*-test, *P* < 0.01) that was more than additive relative to single heterozygotes and approached concentrations seen in *p300*^{KIX/KIX} mice (Fig. 4c). Notably, platelet counts in *c-Myb*^{+/-}*p300*^{+KIX} mice aged 5–6 weeks were similar to those in the single heterozygotes, suggesting that the interaction between c-Myb and the KIX domain of p300 is less crucial in adult thrombopoiesis (data not shown). Thymocyte numbers were not vastly different between wild type and single heterozygotes, but were reduced by about 40% in *c-Myb*^{+/-}*p300*^{+KIX} mice as compared with wild type, suggesting that there is an interaction between c-Myb and the p300 KIX domain in these cells (analysis of variance, *P* < 0.01, *n* = 13–21 per strain; data not shown). Other aspects of *c-Myb*^{+/-}, *p300*^{+KIX} and *c-Myb*^{+/-}*p300*^{+KIX} mice appeared normal (data not shown), except that the single heterozygotes had slightly more platelets than did their wild-type littermates ($(1.52 \pm 0.29) \times 10^9$ for *c-Myb*^{+/-}, and $(1.55 \pm 0.27) \times 10^9$ platelets per ml for *p300*^{+KIX}, *t*-test, *P* < 0.01; Fig. 4c).

When we examined megakaryocyte ploidy in mice aged 5–6 weeks, *c-Myb*^{+/-}*p300*^{+KIX} cells showed a synergistic reduction in DNA content as compared with the single heterozygotes, with fivefold more cells with 8*n*, similar numbers of cells with 16*n*, and

12-fold fewer cells with 32*n* relative to wild type (Fig. 4d, g). *c-Myb*^{+/-} megakaryocytes had a noticeable but less marked 'left shift' (that is, about twofold more 8*n* and twofold less 32*n* cells), whereas *p300*^{+KIX} megakaryocytes seemed to have an intermediate phenotype (Fig. 4d–f). This left shift towards a modal ploidy of 8*n*, which is reminiscent of *p300*^{KIX/KIX} megakaryocytes (Fig. 4h), is abnormal^{19,20}. There was also a significant increase in the percentage of megakaryocytes (CD42d^{hi} cells) in the bone marrow of *c-Myb*^{+/-} and *c-Myb*^{+/-}*p300*^{+KIX} mice as compared with wild-type mice (*t*-test, *P* < 0.01; Fig. 4i), which is consistent with a trend towards megakaryocytosis. Together, these findings suggest that decreased c-Myb-dependent transcription, either by reduction of c-Myb protein (*c-Myb*^{+/-}) or by a transactivation function (*p300*^{KIX/KIX}), results in abnormal megakaryocytopoiesis and predisposes mice to develop thrombocytosis.

Although the *p300*^{KIX/KIX} phenotype does not simply recapitulate the phenotypes of knockout mouse models of CREB, c-Myb or other known KIX-binding proteins, connections between p300 KIX and c-Myb were identified nonetheless. *c-Myb*^{+/-} mice die in embryogenesis owing to a multilineage failure of haematopoiesis, but megakaryocytes are still present, which suggests that any loss of c-Myb function preferentially favours megakaryocytes over other haematopoietic lineages^{15,21}. Whether binding of the p300 KIX domain to c-Myb or CREB is necessary for the development of other haematopoietic lineages affected by mutations in c-Myb (erythroid, myeloid and B cells) or CREB (thymocytes) remains to be tested^{15,16,22}. Notably, the haematopoietic phenotype is specific for *p300*^{KIX/KIX} mice even though previous work has shown that

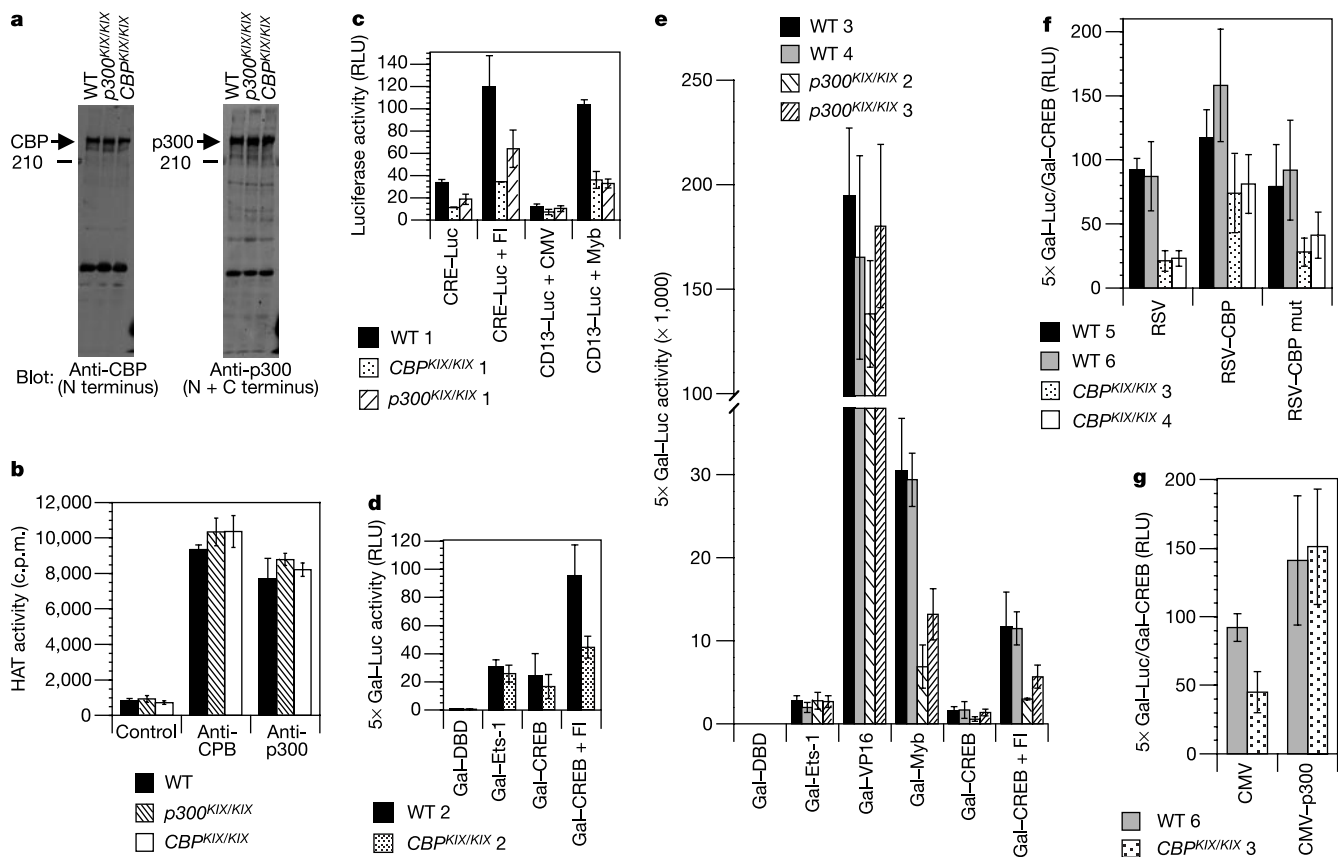


Figure 3 Mutant CBP and p300 affect CREB and c-Myb. **a**, CBP and p300 western blots. **b**, HAT activity in immunoprecipitated CBP and p300 (*n* = 2). **c**, Activity of c-Myb and CREB in *CBP*^{KIX/KIX} and *p300*^{KIX/KIX} MEFs (*n* = 2–4). CD13-Luc is a c-Myb responsive reporter; CMV is the empty vector. FI, forskolin plus isobutylmethylxanthine; RLU, relative light units. **d**, Activity of Gal-CREB, but not Gal-Ets-1, is attenuated in *CBP*^{KIX/KIX} MEFs

(*n* = 2–5). Gal-DBD, Gal4 DNA-binding domain. **e**, Activity of Gal-Myb and Gal-CREB, but not Gal-Ets-1 and Gal-VP16, is affected in *p300*^{KIX/KIX} MEFs (*n* = 3). **f**, **g**, Activity of Gal-CREB is rescued in FI-treated *CBP*^{KIX/KIX} MEFs (*n* = 6, **f**; *n* = 4, **g**). RSV-CBP, CBP; CMV-p300, p300; RSV-CBP mut, KIX-mutant CBP. Means $\pm$ s.d. are shown in **c–g**. WT 1–6, *p300*^{KIX/KIX} 1–3 and *CBP*^{KIX/KIX} 1–4 indicate independent MEF lines.

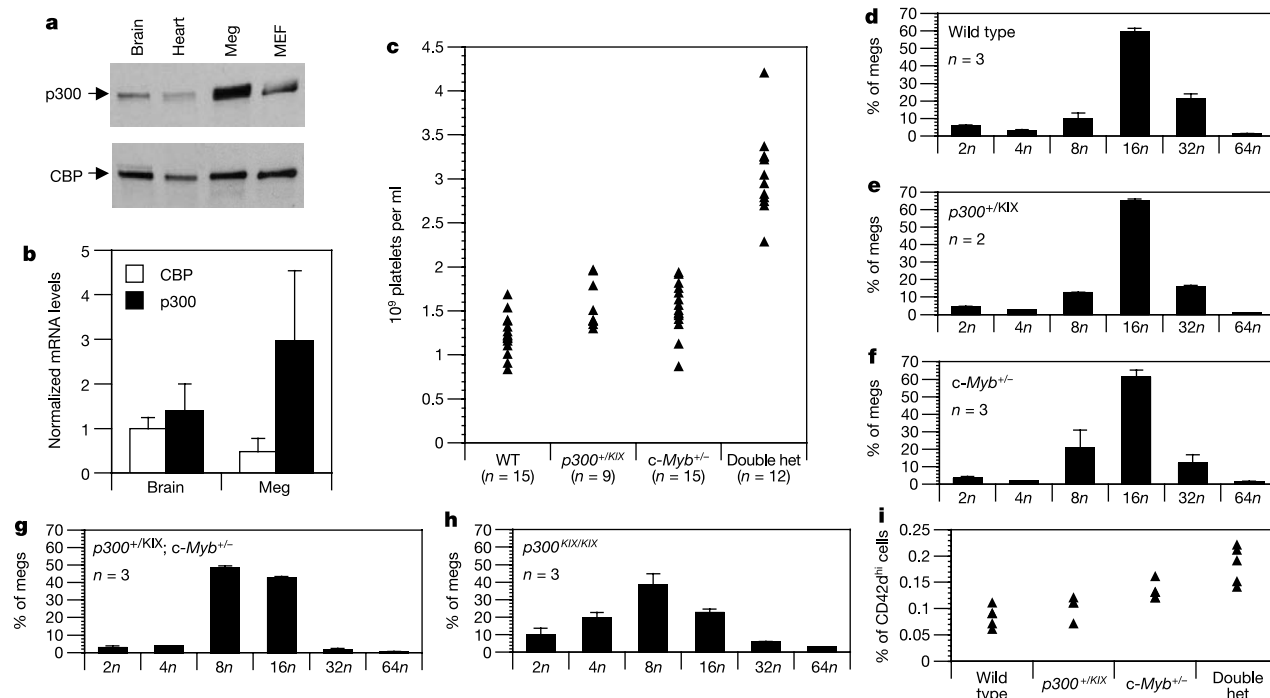


Figure 4 *c-Myb* and *p300* KIX functionally interact to regulate megakaryocytopoiesis. **a**, Western blot of CBP and p300 using wild-type nuclear extracts. Meg, megakaryocyte. Results are normalized to CBP. **b**, Quantitative real-time RT-PCR analysis of *CBP* and *p300* mRNA in wild-type tissues. Shown are the means $\pm$ s.d. relative to *CBP* mRNA in brain (set to 1) after normalization to *GAPDH* mRNA ($n = 12$ or 18). **c**, Platelet counts of

littermates aged 2–3 weeks ($n = 9-15$). Double het indicates $p300^{+/KIX}; c-Myb^{+/-}$ mice. **d–h**, Ploidy analysis of megakaryocytes from littermates aged 5–6 weeks (the $p300^{KIX/KIX}$ mice in **h** were not littermates). Shown are the means $\pm$ s.d. percentages of megakaryocytes ($n = 2-3$). **i**, Percentage of CD42^{dhi} cells (megakaryocytes) in bone marrow ($n = 4-5$).

CBP^{+/-}, but not *p300*^{+/-}, mice tend to develop multilineage defects in haematopoietic differentiation⁴. Taking into account our findings that p300 may be relatively more abundant in an affected lineage, a comparison of the *CBP*^{+/-} and *CBP*^{KIX/KIX} phenotypes implies that other domains besides KIX (or other KIX residues) are limiting for haematopoiesis. Finally, our findings imply that mutations in the KIX domain of p300 and in *c-Myb* may contribute to human haematopoietic diseases, particularly those with abnormal megakaryocytopoiesis and thrombopoiesis. □

Methods

Mice

Targeting vectors for CBP and p300 (Fig. 1d, e) were made from DNA that was isogenic with the E14 ES cells used in this study. We generated point mutations in the KIX exons using PCR with the following primers: CBP, 5'-CATTTACTGCAGGAGAAAATCGCT AAAATACAAAAAGAACTAGAA-3' and 5'-GATTTTCTCCTGCAGTAAATGAGCGTA TTCATCTCTAAAAAGAAAT-3'; p300, 5'-CACCTCTGCAGGAGAAATCGCTAAGAT CCAGAAGGAAGTAA-3' and 5'-GATCTTCTCCTGCAGGAGGTGAGCGTATT CCGCCTTGAAGAAAT-3'. The Hartwell Center at St Jude supplied oligonucleotides and DNA sequencing. Mutations in the KIX exon were confirmed by PCR. Correctly targeted ES cells were confirmed by Southern blot. Transiently expressed Cre recombinase was used to excise *neomycin*^r and the thymidine kinase gene flanked by *loxP* sites (NeoTK) in correctly targeted ES cells; 2'-fluoro-2'-deoxy-5-iodouracil- β -D-arabinofuranoside (FIAU) negative selection was used to enrich for clones that lost NeoTK. We generated chimaeric mice from ES cells by standard methods. KIX mutant mice for initial phenotypic analyses were on a 129Ola/C57BL/6 mixed genetic background; these were comparable to F₁ hybrid KIX mutant mice derived from mating C57BL/6 and 129Ola backcrossed mice. For the *c-Myb*^{+/-} $p300^{+/KIX}$ experiments, *c-Myb*^{+/-} and $p300^{+/KIX}$ parents were maintained on a C57BL/6 background. In bone marrow transplant experiments, 10^6 or 2×10^6 bone marrow cells (varied by experiment) were injected into the tail veins of lethally irradiated (1,100 rad) C57BL/6 mice. We determined Tpo concentrations with an enzyme-linked immunosorbent kit (R&D Systems).

Flow cytometry

Cells were stained with antibodies to CD41, CD45R (B220), CD4, CD8 (PharMingen), IgM and IgD (Southern Biotechnologies) conjugated to fluorescein isothiocyanate (CD41, IgD), phycoerythrin (CD8, IgM), allophycocyanin (B220) or biotin (CD4). Biotinylated

antibody was used with a streptavidin-RED613 conjugate (Invitrogen). We carried out megakaryocyte ploidy analysis as described²³.

Plasmids and antibodies

The reporter and expression plasmids have been described^{8,13,24-26}. Gal-Myb contained residues 186–325 of *c-Myb*, Gal-Ets-1 contained residues 2–210 of Ets-1, and Gal-CREB contained residues 1–283 of CREB. We generated RSV-CBPmut by introducing the Tyr650Ala, Ala654Gln, Tyr658Ala triple mutation into CBP expression plasmid pRC/RSV-mCBP-HA-RK (a gift from R. Goodman) by site-directed mutagenesis. The SV40-Renilla reporter plasmid pRL-SV40 was from Promega. Antibodies were from Santa Cruz (CBP, A22; p300, N15; p300, C20; GST, Z5).

Cell culture and transient assays

We obtained megakaryocytes from the fetal livers of C57BL/6 $\times$ 129Ola F₁ hybrid wild-type mice (E13.5)²⁷. After 5 d of culture, highly enriched primary megakaryocytes were isolated using a bovine serum albumin step gradient (R. Shivdasani, personal communication). We isolated MEFs from E12.5–E14.5 embryos; growth rates were comparable between wild-type and mutant cells. For transient transfection assays, 3×10^4 cells per well were cultured overnight in 24-well plates. Cells were washed with 1 ml of DMEM medium without fetal bovine serum (FBS) or antibiotics. We added 0.25 ml of DMEM containing 2.5 μ l of 2 mg ml⁻¹ Lipofectamine Reagent (Gibco-BRL) and plasmid DNA (0.25 μ g of reporter, 0.25 μ g of expression plasmid, 5 ng of pRL-SV40, 1 μ g of RSV-CBP, 0.25 μ g of CMV-p300) according to the protocols of Gibco-BRL. After 2 h, the DNA/Lipofectamine was removed and 1 ml of DMEM plus 10% FBS without antibiotics was added. We incubated the cells overnight and treated them optionally the next morning with 10 μ M forskolin plus 100 μ M isobutyl-methylxanthine (IBMX) for 6–8 h.

Cell extracts were prepared by washing the cells with PBS followed by *in situ* lysis with 50 μ l of passive lysis buffer (Promega). Dual luciferase assays were carried out according to the manufacturer's protocols (Promega), but with 50% of the recommended volumes. We normalized luciferase activity to Renilla luciferase derived from the cotransfected SV40-Renilla internal control reporter for all experiments. Most data are reported in relative light units relative to the activity of CREB in one line of wild-type MEFs without CBP or p300 overexpression (arbitrarily set to 100). For CBP and p300 rescue experiments, MEFs were maintained on a 3T9 protocol (cells were plated every 3 d to the equivalent of 9×10^5 cells per 6-cm dish), and the empty vector control had equal moles of RSV or CMV expression plasmid, mass balanced with Bluescript SKII DNA (Stratagene).

Nuclear extracts, western blots and HAT assays

Nuclear extracts were prepared by standard methods using buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl and 1 mM EDTA) and buffer C (20 mM HEPES, pH 7.9,

1.5 mM MgCl₂, 420 mM KCl, 25% glycerol and 0.2 mM EDTA) Both buffers contained 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 0.5 µg ml⁻¹ leupeptin and 1 µM pepstatin. Each brain was homogenised in 5 ml of buffer A; nuclear proteins were eluted in 1 ml of buffer C. Extracts were not dialyzed. We carried out western blot transfers of CBP and p300 in cold transfer buffer containing 0.1% SDS for 2 h at 400 mA. HAT assays were done as described, with the nonspecific control antibody GST Z-5 (ref. 13). Western blot analysis of immunoprecipitates following the HAT assays showed that equivalent amounts of CBP or p300 precipitated from mutant and wild-type extracts (data not shown). We used equal amounts of wild-type and mutant total nuclear protein for western blot analysis and HAT assays.

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Probing the free-energy surface for protein folding with single-molecule fluorescence spectroscopy

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Protein folding is inherently a heterogeneous process because of the very large number of microscopic pathways that connect the myriad unfolded conformations to the unique conformation of the native structure. In a first step towards the long-range goal of describing the distribution of pathways experimentally, Förster resonance energy transfer¹ (FRET) has been measured on single, freely diffusing molecules^{2–4}. Here we use this method to determine properties of the free-energy surface for folding that have not been obtained from ensemble experiments. We show that single-molecule FRET measurements of a small cold-shock protein expose equilibrium collapse of the unfolded polypeptide and allow us to calculate limits on the polypeptide reconfiguration time. From these results, limits on the height of the free-energy barrier to folding are obtained that are consistent with a simple statistical mechanical model, but not with the barriers derived from simulations using molecular dynamics. Unlike the activation energy, the free-energy barrier includes the activation entropy and thus has been elusive to experimental determination for any kinetic process in solution.

The basic concepts of our FRET experiment are shown in Fig. 1. A green fluorescent donor dye and a red fluorescent acceptor dye were attached to cysteine residues introduced at the amino and carboxy termini of the cold-shock protein from the hyperthermophilic bacterium *Thermotoga maritima* (CspTm). This protein was selected for study because of its high stability, which makes it tolerant to structural perturbations, and the simplicity of its thermodynamic and kinetic behaviour in ensemble measurements⁵. If a folded CspTm molecule diffuses into the volume illuminated by a focused laser beam, then excitation of the donor dye results in rapid energy transfer to the acceptor dye because the termini are separated by only 1 nm, and most of the fluorescence photons are emitted by the acceptor. On addition of a chemical denaturant the protein unfolds, which results in a larger average distance between the donor and acceptor dyes. Consequently, the energy transfer rate is decreased and the fraction of photons emitted by the acceptor is lower. To help us interpret the results quantitatively, we used a control system consisting of two different lengths of type II polypro-

line helices labelled with the same dyes (Fig. 1). Polyproline provides a rigid spacer between donor and acceptor^{6–8}, which means that the interdy distance is independent of denaturant concentration, whereas all other parameters are expected to vary in the same way that they do in the protein.

Figure 2a and b shows parts of two typical data sets for the polyproline control. For (Pro)₆, the bursts of counts (that is, the detected photons) above background resulting from diffusion of single molecules into the illuminated volume come mostly from the red fluorescing acceptor dye, which shows that FRET is occurring (Fig. 2a). In (Pro)₂₀ there is a larger separation between the dyes (Fig. 1) and, on average, comparable numbers of green and red counts are observed in each burst (Fig. 2b). The apparent FRET efficiency (E_{app}) for each burst is calculated as the ratio of acceptor counts to the sum of acceptor plus donor counts. Figure 2c and d shows the E_{app} distributions for (Pro)₆ and (Pro)₂₀ as a function of the concentration of guanidinium chloride (GdmCl). For (Pro)₂₀, the distribution of E_{app} peaks near 50%. This E_{app} is higher than might be expected from the 6.2-nm polyproline helix, considering that R_0 (the distance at which our dye pair is expected to exhibit 50% transfer) is 5.4 nm. It results from the long flexible linkers of the dyes (Fig. 1), which allow the dyes to approach each other during the fluorescence lifetime of the donor. The additional maximum at $E_{app} \approx 0$ arises from (Pro)₂₀ molecules in which the acceptor dye has been chemically altered by photodestruction, or from (Pro)₂₀ molecules labelled only with the donor dye that were not completely

removed during preparation.

For CspTm, three subpopulations are clearly resolved in the histograms of E_{app} (Fig. 2e), corresponding to folded molecules with large E_{app} , unfolded molecules with intermediate E_{app} , and molecules with $E_{app} \approx 0$ owing to a missing or inactive acceptor, as found for (Pro)₂₀. The finding of only folded and unfolded populations, whose relative proportions change with increasing GdmCl concentration, is exactly what is expected for this protein, which, like unlabelled CspTm^{5,9}, shows two-state behaviour in ensemble equilibrium and kinetic measurements (Supplementary Information). Similarly, two populations have been resolved in single-molecule FRET measurements⁴ of chymotrypsin inhibitor 2 (CI2), which is also known to show two-state behaviour from ensemble measurements¹⁰.

Each E_{app} distribution for CspTm was fit with a sum of one gaussian and two lognormal functions, for the unfolded, folded and donor-only protein peaks, respectively. Figure 3 shows how the means and widths of the E_{app} distributions for (Pro)₂₀ compare with those of the protein. As pointed out previously⁴, resolving the FRET efficiencies for the folded and unfolded subpopulations can expose changes in $\langle E_{app} \rangle$ for the unfolded protein that cannot be extracted from equilibrium ensemble measurements. For unfolded CspTm, $\langle E_{app} \rangle$ clearly increases between 3 and 0 M GdmCl (Fig. 3b). Solvent effects on the dyes can be excluded as a source of the increased FRET efficiency for the unfolded protein, because $\langle E_{app} \rangle$ for (Pro)₂₀ shows almost no change over this range of GdmCl

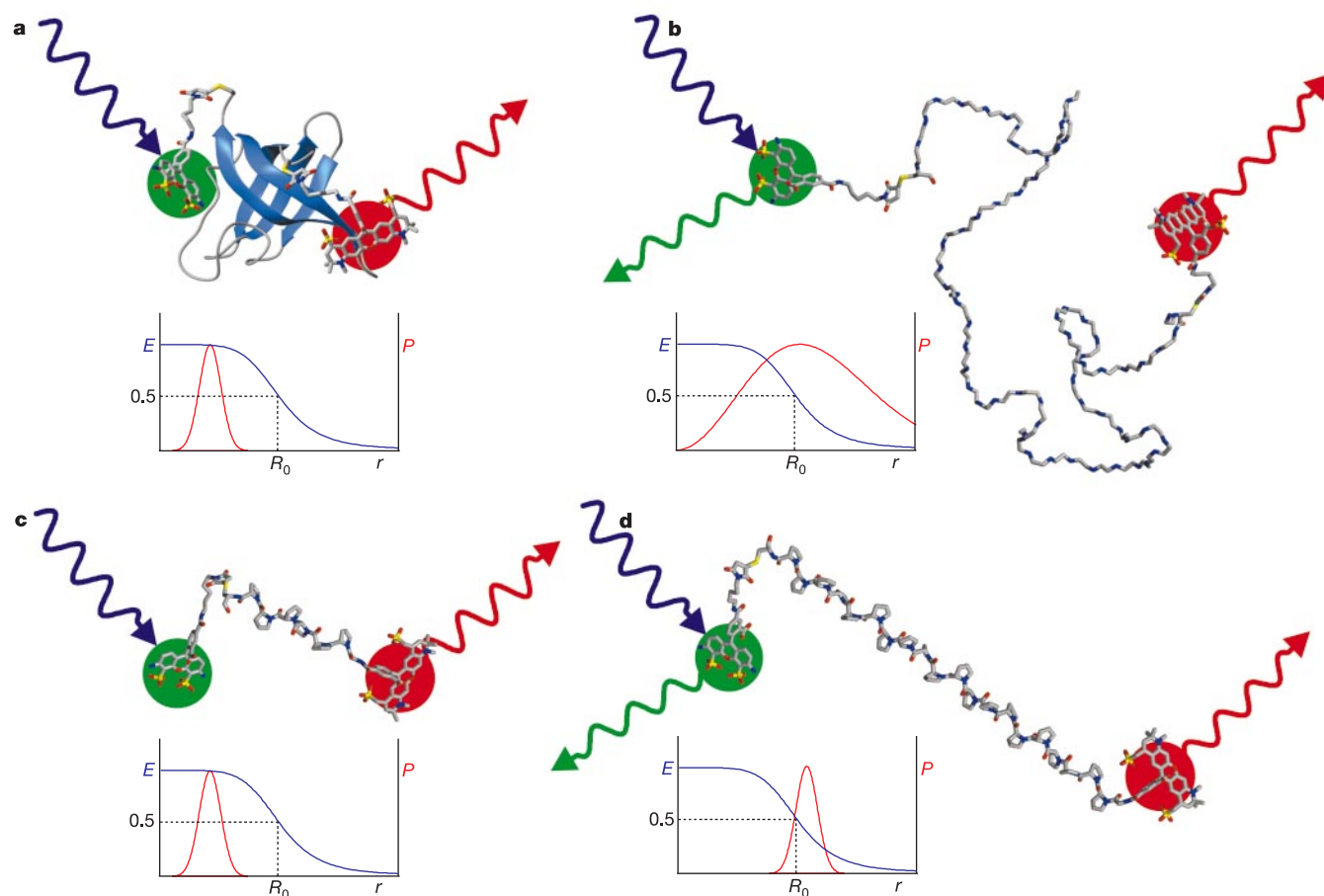


Figure 1 Schematic structures of protein and polyproline helices labelled with donor (Alexa 488) and acceptor (Alexa 594) dyes (using the program MOL/MOL). **a**, Folded CspTm, a 66-residue, five-stranded β -barrel protein (Protein Data Bank accession code 1G6P)³¹; **b**, unfolded CspTm; **c**, (Pro)₆; and **d**, (Pro)₂₀. A blue laser excites the green-emitting donor dye, which can transfer excitation energy to the red-emitting acceptor dye

at a rate that depends on the inverse sixth power of the interdy distance^{1,8}. In each case, the functional form of the FRET efficiency E versus distance (blue curve) is shown, as well as a representation of the probability distribution of distances between donor and acceptor dyes, P (red curve).

concentrations (Fig. 3a). Therefore, we must be observing a decrease in the average end-to-end distance, which indicates collapse to more compact denatured structures at low denaturant concentration. The change in the size of the unfolded protein is qualitatively consistent with predictions of an approximate mean field theory for a weakly hydrophobic sequence¹¹. But the predicted continuous expansion of the chain between 3 and 6 M GdmCl is not observed for CspTm (Fig. 3b) and is also not observed in either ensemble FRET¹² or small-angle X-ray scattering experiments on unfolded cytochrome *c* (ref. 13). The most obvious explanation is that above 3.0 M GdmCl the unfolded polypeptide binding sites become saturated with denaturant molecules.

Important dynamical information is contained in the widths of the E_{app} distributions, that is, the range of FRET efficiencies observed for individual bursts of photons. Notably, the width of the peak corresponding to the unfolded protein is the same to within experimental error as that observed for (Pro)₂₀ (Fig. 3c), in spite of the large difference in flexibility between the two polypeptides. Because the end-to-end distance of polyproline is fixed, the FRET efficiency is nearly the same for all molecules and so a sharp distribution would be expected (Fig. 1). The width of the distribution observed for (Pro)₂₀ arises primarily from fluctuations in E_{app} owing to the small number of photons detected in each burst (see below). By contrast, an unfolded protein has a broad distribution of end-to-end distances (Fig. 1b), which makes the resulting transfer efficiency distributions sensitive to the polypeptide dynamics (Fig. 4). If the chain motion were infinitely slow relative to the observation time t , then every molecule diffusing through the focus would show a different FRET efficiency, resulting in an extremely broad distribution (Fig. 4a). If, in the opposite limit, the chain motion were sufficiently rapid for the unfolded protein

molecule to explore most of its accessible conformational space during the observation time, then the same FRET efficiency would result for every molecule (in the absence of noise and other effects not related to distance, Fig. 4b).

The lack of any width in the E_{app} distributions for the unfolded protein in excess of that observed for (Pro)₂₀ (Fig. 3c) indicates that the end-to-end distance distribution for the unfolded protein does not contribute to the distribution of transfer efficiencies. The unfolded protein must therefore be reconfiguring fast relative to t . The relaxation time of the FRET efficiency autocorrelation function, τ_E , is given by $t(\sigma_{app}^2 - \sigma_0^2)/2\sigma_E^2$ for $\tau_E < t$ (ref. 14), where σ_{app}^2 is the variance in E_{app} for the unfolded protein, σ_0^2 is the variance owing to noise and other effects not dependent on the interdy distance, and σ_E^2 is the variance of the FRET efficiency resulting from the underlying equilibrium distance distribution (Fig. 4a). For a gaussian chain with a mean squared end-to-end distance $\langle r^2 \rangle = R_0^2$, this relationship yields a polypeptide reconfiguration time of $\tau_0 = 9.8t(\sigma_{app}^2 - \sigma_0^2)$. Equating σ_0^2 with the variance for (Pro)₂₀ and recognizing that, to within experimental error, σ_{app} cannot be more than 25% larger than σ_0 (Fig. 3c), the maximum value for τ_0 consistent with our observations is $t/40$. Variation in the time intervals used in the data collection indicates that the average t is about 1 ms, so that the upper limit for τ_0 is about 25 μ s.

Our interpretation of the E_{app} distributions is different from those given previously^{3,4}, in which the additional width beyond that predicted from shot noise (the variation in count rates about fixed

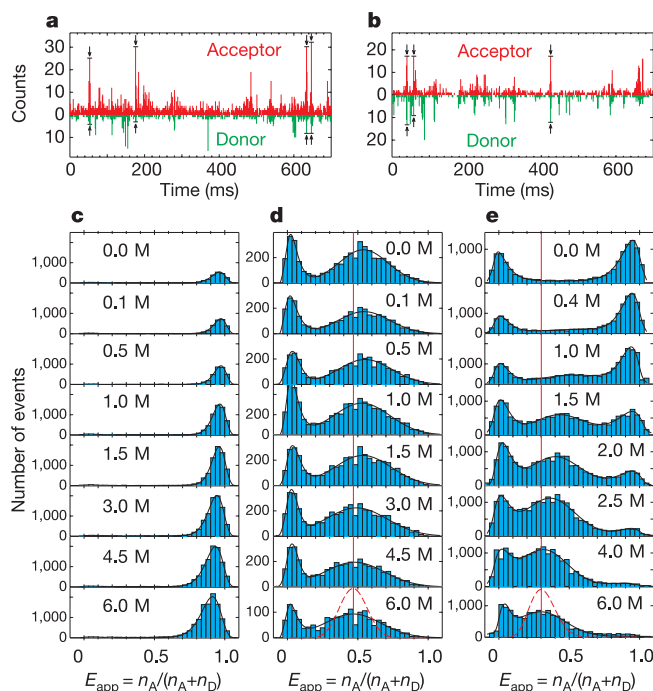


Figure 2 FRET trajectories and histograms. **a, b**, Donor (green) and acceptor (red) channel time traces using 1-ms bins for labelled (Pro)₆ (**a**) and (Pro)₂₀ (**b**). Arrows indicate photon bursts for which the sum of the counts in the two channels is greater than 25. **c–e**, Histograms of measured FRET efficiencies (E_{app}) at various GdmCl concentrations for labelled (Pro)₆ (**c**), (Pro)₂₀ (**d**) and CspTm (**e**). The black curves are the best fits to the data using lognormal and/or gaussian functions. The red dashed curves were calculated from the β -distribution¹⁵ $P(E_{app}) = E_{app}^{(n_A)} (1 - E_{app})^{(n_D)}$, where $\langle n_A \rangle$ and $\langle n_D \rangle$ are the average number of detected acceptor and donor photons in the significant bursts.

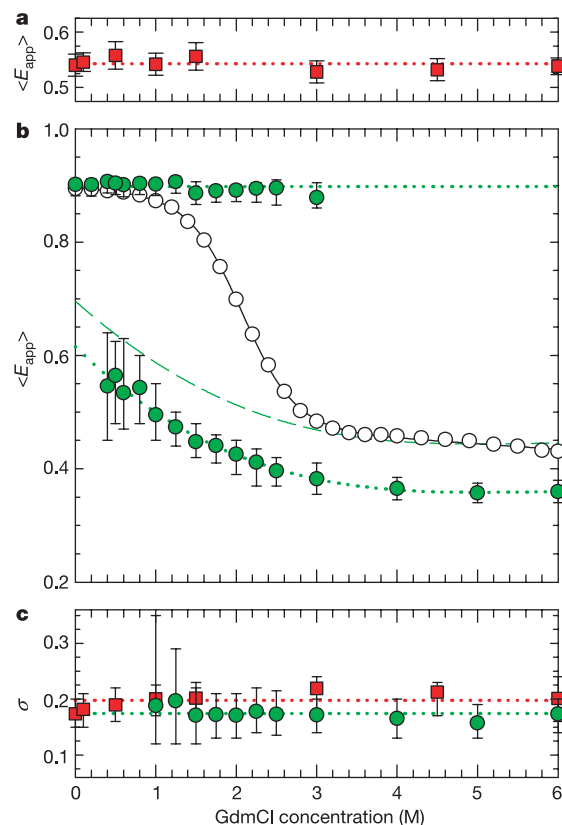


Figure 3 Dependence of the means and widths of the measured FRET efficiency (E_{app}) on the concentration of GdmCl. **a**, $\langle E_{app} \rangle$ for (Pro)₂₀. **b**, Single molecule mean values (filled circles), ensemble FRET efficiencies (open circles), and associated two-state fit (unbroken curve) for CspTm. The dotted curve is a third-order polynomial fit to the unfolded protein data that was matched (dashed curve) to the ensemble data between 4 and 6 M GdmCl. **c**, Standard deviations (σ) taken from the gaussian fits to the (Pro)₂₀ data (squares) and unfolded CspTm data (circles) in Fig. 2. Error bars indicate uncertainty in the fits.

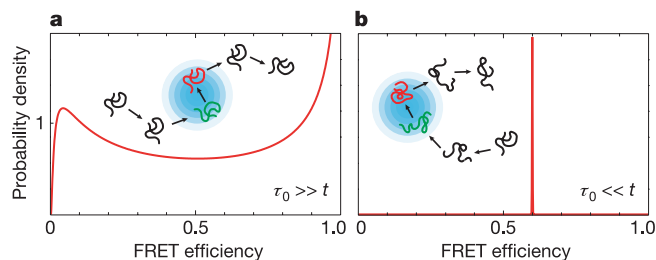


Figure 4 Two limiting cases for polypeptide dynamics in experiments on freely diffusing molecules. **a**, If the end-to-end distance of the protein does not change during the observation period in the illuminated volume (blue), then the distribution of transfer efficiencies reflects the equilibrium end-to-end distance distribution of the molecules and consequently results in a very broad probability distribution of FRET efficiencies, shown here for a gaussian chain (red curve, see Supplementary Information). **b**, If the molecule reconfigures fast relative to the time it takes to diffuse through the illuminated volume, then the FRET efficiency averages completely and (in the absence of shot noise and other broadening effects) is the same for every molecule.

means due to the discrete nature of the signals; ref. 15 and Fig. 2d, e) was attributed to fluctuations of interdy distances on a timescale comparable to or slower than the observation time. Although the possible pitfalls were recognized^{3,4,15}, Förster's equation, $E = 1/(1 + r^6/R_0^6)$ (ref. 1), was used in those studies to transform the E_{app} histograms for the folded and unfolded populations into histograms of interdy distances, r . But E_{app} widths very similar to those of unfolded CI2 (ref. 4) were observed previously for double-stranded DNA labelled with the same donor and acceptor dyes¹⁶. Consequently, it is unlikely that there is a significant contribution to the E_{app} widths for CI2 from a distribution of distances, in agreement with our interpretation of the widths measured for unfolded CspTm. Therefore, the widths in excess of shot noise of the E_{app} distributions for CspTm and CI2 must be caused by other sources, such as nonrandom photon emission intervals resulting from triplet state formation or intensity variation across the focal volume. Quantifying these contributions to the width of the E_{app} distributions will require detailed experimental and theoretical investigation, but this issue does not affect any of the conclusions reached below.

The finding of a reconfiguration time less than 25 μ s can be related directly to important theoretical considerations in protein folding. Extensive studies suggest that essential features of the dynamics of folding can be captured by describing the process as diffusion on a low-dimensional free-energy surface. The free energy as a function of global coordinates, such as the fraction of native inter-residue interactions^{17–19}, contains the depths and average properties of the wells that correspond to thermodynamic states, and the positions and heights of the free-energy barriers separating these states which dictate the rates. Conventional equilibrium and kinetic studies yield the free-energy differences between the thermodynamic states, and the rates connecting them, but not the heights of the free-energy barriers. But Kramers theory, which accurately reproduces the kinetics for simplified representations of proteins^{20–22}, shows how the free-energy barrier height can be obtained if the polypeptide reconfiguration time is known. Kramers equation²³ for the folding time τ_f is

$$\tau_f = \frac{2\pi\omega_{\min}\tau_0}{\omega_{\max}} \exp\left(\frac{\Delta}{k_B T}\right) \approx 2\pi\tau_0 \exp\left(\frac{\Delta}{k_B T}\right) \quad (1)$$

where $\omega_{\min}$ and $\omega_{\max}$ are, respectively, the frequencies that characterize the curvature of the (one-dimensional) free-energy surface in the harmonic well of the unfolded state and at the barrier top, Δ is the height of the folding free-energy barrier, k_B is Boltzmann's constant, T is the absolute temperature, $\tau_0 (= k_B T / m\omega_{\min}^2 D)$ is the

reconfiguration time in the unfolded well, and D is the diffusion constant for motion along the coordinate. Assuming the simplest case in which $\omega_{\min} \approx \omega_{\max}$, as found in lattice²⁰ and off-lattice models²¹, we can determine Δ from τ_f and τ_0 . A lower bound of $4k_B T$ is obtained from the upper limit on τ_0 of 25 μ s (calculated above) and the corresponding ensemble folding time of $\tau_f = 12$ ms at 20 °C (at 0 M GdmCl) for the dye-labelled protein from stopped-flow kinetic measurements (Supplementary Information). An upper bound can be estimated from the reconfiguration time for a gaussian chain, which is simply $\langle r^2 \rangle / 3D$, where $\langle r^2 \rangle \approx 22$ nm² is the mean-squared end-to-end distance for the unfolded protein that can be calculated from $\langle E_{app} \rangle \approx 0.7$ in the absence of denaturant (Fig. 3b and Supplementary Information), and D is the relative end-to-end diffusion constant. Using $D < 1.7 \times 10^{-6}$ cm² s⁻¹ from a study of end-to-end contact rates in disordered peptides²⁴, $\tau_0 > 40$ ns and $\Delta < 11k_B T$. We regard this value of D as an upper limit because it has been determined for peptides containing about 30% glycines; it is expected to be smaller for CspTm because of side chain interactions and the smaller fraction of glycines (15%) in the protein sequence.

Using different and less rigorous arguments, estimates of the pre-exponential factor in equation (1)—that is, $2\pi k_B T / m\omega_{\min}\omega_{\max}D$ —have ranged from 0.05 μ s (ref. 25) to 1 μ s (ref. 26) for a generic small protein, as compared with 0.3 μ s to 0.2 ms for the specific protein studied here. The final result is that we have placed bounds on the free-energy barrier to folding of $11k_B T > \Delta > 4k_B T$. Together with the (viscosity-corrected) activation enthalpy for folding of $5k_B T$ (Supplementary Information), these bounds on the free-energy barrier correspond to an activation entropy between $-6k_B$ and $+1k_B$. The small activation entropy presumably results from cancellation of two large contributions upon forming the transition state ensemble—the entropy loss from ordering the polypeptide and the entropy gain from forming hydrophobic contacts.

The determination of bounds on a free-energy barrier height provides a previously unavailable benchmark for theoretical free-energy surfaces²⁷. Although there are no detailed theoretical calculations for CspTm as yet, two other cold-shock proteins, CspA and CspB, that have the same structure and folding rates as CspTm have been studied. Using a simple statistical mechanical model that considers only inter-residue interactions present in the folded structure, free-energy barriers of $15k_B T$, $11k_B T$ and $7k_B T$ have been calculated for CspB as the allowed number of native stretches of polypeptide in the molecule was increased from one to two to three, respectively²⁸. Our experimental results are, not surprisingly²⁷, inconsistent with the single-sequence approximation but are consistent with double- and triple-sequence approximations. In contrast, calculations of a free-energy surface derived from molecular dynamics simulations that also only consider native interactions in an α -carbon representation of CspA show no free-energy barriers under folding conditions²⁹. In addition, the radius of gyration of the simulated unfolded CspA protein at the folding temperature is only 1.4 nm (where a barrier exists but is only $1.5k_B T$), compared with 2.3 nm calculated from $\langle E_{app} \rangle$ for unfolded CspTm at the midpoint concentration of denaturant. The free-energy surface for CspA calculated from all-atom molecular dynamics simulations at room temperature also predicts the absence of a barrier²⁹, whereas the observation of exponential folding kinetics³⁰ for CspA suggests a free-energy barrier crossing, as found for CspTm.

Note added in proof: C. L. Brooks (ref. 29) has communicated to us that the calculated free energy surface for an α -carbon representation of CspB is more consistent with our experimental results, showing a barrier under folding conditions of about $1k_B T$ and a radius of gyration of 2.0 nm for the unfolded state at the folding temperature. $\square$

Methods

Synthesis and labelling of CspTm and polyproline

Cysteine residues were introduced at the N terminus after Met 1 and at the C terminus by site-directed mutagenesis to provide functional groups for the specific attachment of the dyes. To exclude potential complications owing to proline *cis-trans* isomerization in CspTm, Pro 57 was replaced with glycine. We carried out expression and purification as described³¹. Dye labelling was carried out by procedures described by the manufacturer (Molecular Probes). Alexa Fluor 488 maleimide was reacted with the protein, and singly labelled protein was separated from unlabelled and doubly labelled protein by ion exchange chromatography (MonoQ HR 5/5, Amersham Pharmacia). The fractions containing singly labelled CspTm, as confirmed by electrospray ionization mass spectroscopy, were labelled with Alexa Fluor 594 maleimide. Doubly labelled protein was again separated from singly labelled protein by ion exchange chromatography.

We synthesized polyproline peptides containing 6 and 20 prolines using standard FastMoc chemistry, and incorporated a C-terminal cysteine and an N-terminal glycine as reactive groups for dye labelling. After cleavage and HPLC purification, fractions containing the pure peptide, as confirmed by mass spectroscopy, were labelled with Alexa Fluor 488 maleimide. Singly labelled peptide was purified by size-exclusion chromatography (Amersham Pharmacia) and labelled with Alexa Fluor 594 succinimidyl ester. Doubly labelled peptide was again purified by size-exclusion chromatography.

For single-molecule experiments, samples of labelled protein or peptide were diluted to a concentration of 75 pM or 38 pM, respectively, in 50 mM sodium phosphate (pH 7) plus 0.01% Tween 20 to prevent surface adhesion of the polypeptides. We carried out ensemble experiments on a spectrofluorometer (Spx Fluorolog 2) under identical buffer conditions at protein concentrations of 10 nM. Sequanal Grade GdmCl (Pierce) was used for denaturation experiments. Steady-state polarization measurements of the attached dyes resulted in anisotropies between 0.06 and 0.09 for all samples, indicating sufficient rotational averaging during the fluorescence lifetime of the dyes to justify using $\kappa^2 = 2/3$ (ref. 4). We calculated¹ a value of $R_0 = 5.4$ nm from the donor emission and acceptor absorption spectra of singly labelled CspTm, and a donor fluorescence quantum yield of 0.5, which was measured relative to the manufacturer's quantum yield for the free dye. The raw data shown in Fig. 2 were corrected for the refractive index change with increasing GdmCl concentration, according to the n^{-4} dependence of Förster theory¹, to determine the values in Fig. 3.

Confocal fluorescence microscope

Observations of single-molecule fluorescence were made using a custom confocal optical system with a 1.4 NA $\times 100$ microscope objective (Nikon CFN Plan Apo 85025) and the 488-nm line of an argon ion laser (Lexel 95-5) as an excitation source. Sample fluorescence passed through a 100- μ m diameter pinhole in the image plane of the objective. The fluorescence was separated into donor and acceptor components using a dichroic mirror (Omega 560DCLP), and two final filters (Omega 525AF45 for the donor and Omega 600ALP for the acceptor). Each component was focused onto a photon-counting avalanche photodiode (PerkinElmer Optoelectronics SPCM-AQR-15), and output pulses were collected in intervals of 1 ms. To minimize saturation effects owing to excitation of the donor dye before the acceptor dye had returned to the ground state, we reduced the laser intensity to an acceptable level on the basis of power dependence experiments.

Data reduction

Background (usually between 0.5 and 2 counts), obtained in each experiment from independent measurements of solutions without labelled samples, was subtracted from the counts in each 1-ms interval. The signal was considered significant if the sum of counts in the donor and acceptor channels was greater than 25, thereby ensuring that no more than one burst per thousand was due to background. We combined significant signals from adjacent intervals, because the probability that they arose from different molecules was negligible. Finally, the sums of donor counts (n_D) and acceptor counts (n_A) for each burst were used to compute an apparent FRET efficiency using the relation $E_{app} = n_A / (n_A + \gamma n_D)$, where $\gamma = (\phi_A \eta_A) / (\phi_D \eta_D)$ is a factor that corrects for the difference in the fluorescence quantum yields of the acceptor (ϕ_A) and the donor (ϕ_D) (in the absence of acceptor), as well as differences in the detection efficiencies of the donor and acceptor channels of the instrument, η_D and η_A , respectively. Comparison of the single-molecule instrument with a detector-calibrated fluorometer was made using solutions of the protein singly labelled with donor and acceptor dyes at concentrations that resulted in equal optical densities at 488 nm (100 nM and 2.2 μ M, respectively). These experiments showed that at the laser intensities used in the single-molecule measurements γ is close to 1, the value we therefore used in our analysis. Slight deviations of γ from 1 were observed with increasing radiant flux.

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These notes are compiled in the Nature office from information provided by the manufacturers.

Bioinformatics: Bringing it all together

technology feature

Forget test tubes, petri dishes and pipettes. One of the few pieces of equipment that can be honestly labelled ubiquitous in biology today is the computer. Bioinformatics — the development and application of computational tools to acquire, store, organize, archive, analyse and visualize biological data — is one of biology's fastest-growing technologies.

Biologists at the bench studying small networks of genes want user-friendly tools to analyse their results and help them to plan experiments. They need accessible interfaces that allow them to search databases, and compare their data with those of others (see 'Genome analysis at your fingertips', below).

At the other end of the spectrum, researchers analysing whole genomes, and drug-discovery companies mining the genome for drug targets, want high-throughput analysis tools to accelerate genome annotation and extract information from databases in more efficient and sophisticated ways.

All of those involved want more

integration — integration of data across the hundreds, if not thousands, of different databases, and visual integration of data to aid interpretation. "The key to bioinformatics is integration, integration, integration," says bioinformatics expert Jim Golden at Curagen spin-off 454 Corporation in Branford, Connecticut. "To answer most interesting biological problems, you need to combine data from many data sources," agrees Russ Altman, a biomedical informatics expert at Stanford University. "However, creating seamless access to multiple data sources is extremely difficult."

Standard currencies

One of the most insidious problems is the lack of standard file formats and data-access methods. But attempts to standardize them are gaining momentum. One success is the distributed annotation system (DAS), a standard protocol developed by Lincoln Stein at Cold Spring Harbor Laboratory in New York and his colleagues. "It's a simple solution to a simple but obvious problem," says Stein. "There was no standard way of exchanging sequence annotations."

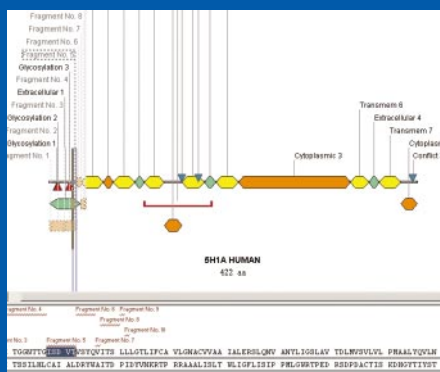
DAS allows one computer to contact multiple servers to retrieve and integrate dispersed genomic annotations associated with a particular sequence, such as predicted introns and exons from one server and corresponding single-nucleotide polymorphisms (SNPs) from another. It handles the annotations as elements associated with a particular stretch of genomic sequence and so enables users to obtain a picture of that genome segment with all of its associated annotations. Many providers of genome data, including WormBase, FlyBase, the Ensembl server run by the European Bioinformatics Institute (EBI) and the Sanger Institute near Cambridge, UK, and the genome browser at the University of California, Santa Cruz, are currently running DAS servers.

Reckoning that data providers will never agree on a universal standard for representing data, building database interfaces or writing access scripts, Stein thinks that web services such as DAS are the best route to interoperability. Data providers only have to agree on a small set of standards that define how their data and

GENOME ANALYSIS AT YOUR FINGERTIPS

The working biologist now has an enormous number of options when it comes to bioinformatics tools. On one hand, there is a lot of free high-quality software in the public domain. On the other, researchers can buy commercial products offering added features, such as programs to streamline sequential tasks, to access proprietary databases and to enhance data security. And because software producers realize that users' needs change and their products will rarely be used in isolation, flexibility and modularity are on the rise.

An important trend has been the increasing integration and sophistication of tools available to non-experts. A wide range of user-friendly packages incorporating tools for nucleotide and protein sequence analysis are available from companies such as MiraiBio, a Hitachi Software Engineering subsidiary based in Alameda, California; DNASTAR in Madison, Wisconsin; InforMax in Bethesda, Maryland; and Accelrys in San Diego, California. On the non-commercial side, the Biology WorkBench maintained by the Supercomputer Center at the University of



InforMax's BioAnnotator uses locally stored databases to find protein motifs.

California, San Diego, is particularly popular, offering more than 80 bioinformatics tools to more than 10,000 registered users. "It's a one-stop-shop for doing a lot of things," says lead developer Shankar Subramaniam. "You can be sitting in front of any type of computer; as long as you have a web browser, you can access it."

Software has also become more user-friendly. Back in the early 1990s, users of the GCG Wisconsin package, the grandfather of molecular-biology packages (now sold by Accelrys), had to work with UNIX-based systems. Although these systems are still preferred by some, users can now point-and-click their way through a wide range of tasks on ordinary desktop computers.

Another trend is the increased integration of data analysis with experimental design. The needs of bench scientists don't always coincide with those of professional bioinformaticians producing tools for whole-genome analyses. Genome projects require programs that can efficiently, if not very accurately, process huge amounts of sequence data, but the biologist in the lab is often interested in studying small sets of genes and their products with

tools are presented to the outside world.

And a 'registry' can keep track of which data sources implement which services. Scripts for retrieving a particular type of data or operation consult the registry, as they would an address book, to determine which data sources to query. A project of this type is BioMOBY, led by Mark Wilkinson at the National Research Council in Saskatoon, Canada. BioMOBY will be a powerful exploration tool, he says, because apart from answering database queries, it will discover cross-references to other relevant data and applications. Betting on BioMOBY's potential, several groups are encouraging its development. "At the moment, we have the support of almost all of the model organism databases," says Wilkinson.

Another indicator of the widespread desire for interoperability is the incorporation in February 2002 of the Interoperable Informatics Infrastructure Consortium (I3C). With 14 member organizations — including Sun Microsystems of Santa Clara, California; IBM of White Plains, New York; Millennium Pharmaceuticals and the Whitehead Institute for Biomedical Research, both in Cambridge, Massachusetts — I3C is not a standards body, but aims to develop and promote the adoption of common protocols.

To integrate the current set of non-standardized databases, researchers are relying on two main strategies: warehousing

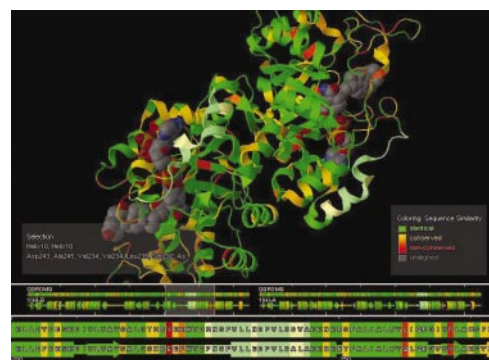
and federation. A warehouse is a central database where data from many different sources are brought together on one physical site. Entrez, the widely used search-and-retrieval system developed by the US National Center for Biotechnology Information in Bethesda, Maryland, is an example.

Access all areas

A popular tool is SRS produced by LION Bioscience of Heidelberg, Germany, which facilitates access to a wide range of biological databases using a warehouse-like strategy. SRS is used in the online genome portals maintained by Celera Genomics in Rockland, Maryland, and Incyte Genomics in Palo Alto, California, and is the core technology of tools sold by LION.

Federation, on the other hand, links different databases so that they appear to be unified to the end-user but are not physically integrated at a common site. A query engine takes a complicated question requiring access to multiple databases and divides it into subqueries that are sent to the individual databases. The answers are then reassembled and presented to the user. Aventis Pharmaceuticals in Strasbourg, France, for example, has adopted IBM's DiscoveryLink federating software to aid collaboration between its biologists and chemists in drug development.

Which approach to use and when is much debated. "Updating and maintaining



Structure prediction: modelling a sequence homolog in LION's SRS 3D.

local copies of external data collections in a warehouse is a major task," says bioinformatician Rolf Apweiler at the EBI's lab in Hinxton, UK. Federation avoids this because the data are accessed directly from the original source. But the bioinformatics databases you want to query must be accessible for programmatic queries over the Internet, and most are not, says Peter Karp, director of the bioinformatics research group at the non-profit research institute SRI International in Menlo Park, California. "It's like installing a state-of-the-art telephone exchange in a village without telephones."

Several projects combine the two approaches. On the industry side, IBM has set up a partnership with LION to

LION BIOSCIENCE

very high precision. Last month, for example, InforMax released GenomBench, a tool that allows users to predict the structure of genes and their splice variants, progressively refine these predictions, and then design experiments to validate them. "It's an interactive tool that can work with researchers not just to analyse the data they have, but to design the right experiment to resolve ambiguities in the data," says Steve Lincoln, senior vice-president of life-science informatics at the company.

Others are hooking up their software to catalogues of reagents. As just one example, the genome browser run by the University of California, Santa Cruz, is being used in a collaboration with the National Cancer Institute in Bethesda, Maryland, to identify new genes to expand, and ultimately complete, the Mammalian Gene Collection — a set of cDNA clones of expressed genes for human and mouse. The browser will be linked to the collection's website, so that users can go straight from analysing an electronic representation of a gene to ordering a clone.

A key trend in the development of commercial products is the emergence of workflows, automated chains of operations that can dramatically increase analysis throughput. For example, software producer geneticXchange of Menlo Park, California, recently demonstrated a workflow that sorts gene-expression data generated by microarrays, looks up the accession numbers that identify the selected genes, collects sequence information from the US National Center for Biotechnology Information's UniGene database, gathers

annotation information from the LocusLink website, and goes to Medline to assemble a list of relevant references. "You just hit a button and it does what might take a biologist 600 hours to do, in about five hours," says Mark Haselup, chief technical officer for the company.

Some commercial products are valuable because they're linked to otherwise unavailable proprietary data. One of the main selling points of the Celera Discovery System, for example, is the access it provides to the biotech firm's high-quality human and mouse genome annotations. Unlike many other collections of annotations, a high proportion of Celera's have been generated by manual curation (see 'Putting a name on it', overleaf).

Commercial products often provide greater security for those who don't wish to manipulate their unpublished or unpatented results openly over the Internet. Although some public sites offer a degree of security, commercial packages usually have more protection options and can be operated behind a firewall.

But the recurrent theme in the design of bioinformatics tools is the trend towards increased integration. The Discovery Studio Gene package recently launched by Accelrys is a case in point. "Results are put into a project database that has the ability to be accessed by a set of applications that span both chemistry and biology," says Scott Kahn, senior vice-president of life science at Accelrys. "We set up the ability to collaborate between domains." **M.C.** Biology WorkBench workbench.sdsc.edu

integrate DiscoveryLink with SRS. Particularly ambitious is the public-domain Integr8 project led by Apweiler. His team aims to bring together some 25 major databases spanning a broad range of molecular data, from nucleotide sequences to protein function. "We're trying to make an integrative layer on top of it all so that you can easily zoom in on the sequence data linked to the gene, and then go to the genomic data, to the transcriptional data and to the protein sequences. You'll have a sort of magnifying glass," says Apweiler.

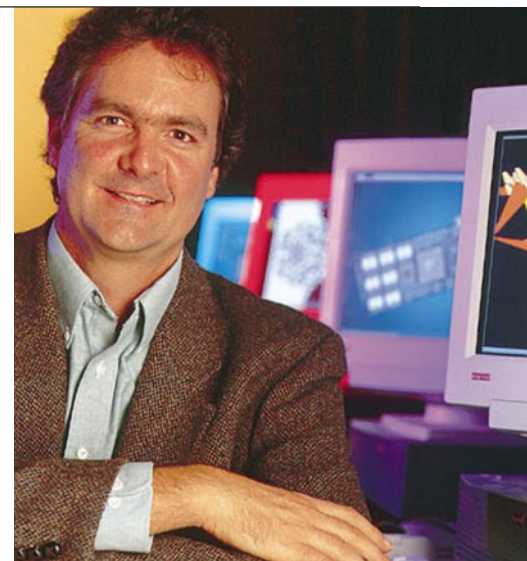
Knowledge is power

Smart systems that can answer complicated questions about different sorts of data are also on the move. "A knowledge base is a fancy word for a database that allows you to do really sophisticated queries," says bioinformatician Mark Yandell at the University of California, Berkeley. Such databases often rely on vocabularies known as 'ontologies' (see 'Putting a name on it', below) combined with frame-based systems, a way of representing data in computers as objects within a hierarchy. One frame, for example, could be called 'protein', with slots describing its relationships to other concepts, such as 'gene name', or 'post-translational modifications'. So when a user asks a question about a protein, frames make it easy to retrieve the name of the corresponding gene and the modifications

the protein can undergo. If the user asks for literature references, ontologies make it possible to retrieve not only articles that include the protein name but also those about related genes or processes.

The Genome Knowledgebase, a collaborative project between Cold Spring Harbor Laboratory, the EBI and the Gene Ontology Consortium, will have, among other capabilities, the ability to make connections between disparate genomic data from different species. "We store things specific to a species but allow a patchwork of evidence from different species to weave together," says Ewan Birney, a bioinformatician at the EBI. So when users pose questions about a biological process, they will get answers that incorporate knowledge collected from various model organisms.

Knowledge bases are being developed for a wide variety of topics, but some researchers are sceptical about their future. Information scientist Bruce Schatz of the University of Illinois at Urbana-Champaign, for example, thinks that ontologies require too much expert effort to generate and maintain. "All ontologies are eventually doomed," he says. Instead, he favours a purely automated process of knowledge generation, such as concept-switching, which relies on analysing the contextual relationships between phrases to identify underlying concepts. Concept-switching algorithms, for example, allow



David Haussler: putting the picture together.

users to start with a general topic, such as mechanosensation, and explore its 'concept space', zeroing in on specific terms such as the mechanosensory genes of a particular species.

Visualizing the genome

An essential component of bioinformatics is the ability to visualize retrieved data, especially complex data, in ways that aid their interpretation. "Integration and visualization are actually very closely related, because after you integrate

R.R. JONES

PUTTING A NAME ON IT



BILL GEDDES

Lincoln Stein: bridging the gap.

A chasm separates sequence data from the biology of organisms — and genome annotation will be the bridge, says Lincoln Stein, a bioinformatics expert at Cold Spring Harbor Laboratory in New York. Spanning three main categories — nucleotide sequence, protein sequence and biological process — annotation is the task of adding layers of analysis and interpretation to the raw sequences. The layers can be generated automatically by algorithms or meticulously built up by experts in the hands-on process of manual curation.

Because manual curation is time-consuming and genome projects are generating data, and even changing data, at an extraordinary pace, there is a strong motive to shift as much of the burden as possible to automated procedures. A major task in the annotation of genomes, especially large ones, is finding the genes. There are numerous gene-prediction algorithms that combine statistical information about gene features, such as splice sites, or compare stretches of genome sequence to previously identified coding sequences, or combine both approaches. A new type of algorithm, called a dual-genome predictor, uses data from two genomes,

to locate genes by identifying regions of high similarity.

Each algorithm has its strengths and limitations, working better with certain genes and genomes than with others. The GENSCAN gene-predicting algorithm, developed by Chris Burge at the Massachusetts Institute of Technology, has become a workhorse for vertebrate annotation and was one of the algorithms used in the landmark publications of the draft human genome sequence. FGENESH, produced by software firm Softberry of Mount Kisco, New York, proved particularly useful for the Syngenta-led annotation of the rice genome sequence.

Good data preparation is also important. "A lot of the magic happens in the environment, not the algorithm," says Ewan Birney a bioinformatician at the European Bioinformatics Institute (EBI) in Hinxton, near Cambridge, UK. "People often focus on the whizzy technology to the detriment of the real smarts, which happen in the sanitization of data to present them to a hard-core algorithm." Data sanitization includes steps such as masking repetitive sequences, which can interfere with an algorithm's performance.

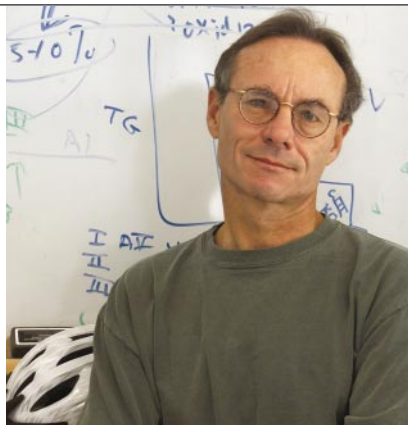
All current large-scale efforts involve a combination of automatic and manual approaches. "For me it's quite clear that they can only be complementary," says Rolf Apweiler at the EBI, who leads annotation for the major protein databases SWISS-PROT and TrEMBL. "You can't automate anything without having

information, the first thing you want to do is display it," says Altman. "They're both parts of the issue of taking information that's perfectly happy in a computer and turning it into information that a user is happy digesting cognitively."

Genome browsers are particularly powerful, as they provide a bounded framework, the genome sequence, onto which many different types of data can be mapped. The University of California, Santa Cruz, for example, maintains a browser where users can simultaneously view the locations of SNPs, predicted genes and mRNA sequences along a chosen genome stretch. "It's all about linking," says principal investigator David Haussler. "It's about having it all at your fingertips."

Tools that compare genomes from different species are also proving their worth. The VISTA project, developed and maintained by the Lawrence Berkeley National Laboratory in Berkeley, California, allows biologists to align and compare large stretches of sequence from two or more species. "It gives you a graphical output where you see peaks of conservation and valleys of lack of conservation," says Edward Rubin, one of VISTA's developers.

Spotfire of Somerville, Massachusetts, sells software that can transform all sorts of data into images. Using Spotfire's DecisionSite, researchers at Monsanto in St Louis, Missouri, represented as a 'heat map' the results of complex experiments



Edward Rubin takes a graphical view.

that tracked changes in the expression of thousands of genes and the concentrations of numerous metabolites during maize development. It helped them to link the expression of certain genes to the presence or absence of particular amino acids. "A lot of times it's through comparisons and comparisons and comparisons that researchers see an interesting trend," says David Butler, vice-president of product strategy at Spotfire.

Biologists are moving closer to their dream of data integration. But open issues remain. Schatz worries that if public support doesn't increase, industry may come to dominate the field, providing suboptimal solutions for scientists. "If a Celera-like company starts doing this kind of activity and they get bought by Microsoft, which is

an entirely possible activity in the world at large, then it will be too late. And then scientists will get whatever the major customers of Microsoft want," he says.

But Celera's director of scientific content and analysis, Richard Mural, advocates a centralized, industry-based solution to integration and genome annotation. He notes that there are few rewards for academic researchers for working on such problems, and their focused interests can be hard to reconcile with a global approach. "To really get it done quickly and well, I think the commercial may be a stronger model," he says.

However these issues are resolved, the road ahead looks bright. "Ninety-nine percent of bioinformatics is new stuff," says Haussler. "It's an enormous frontier."

Marina Chicurel is a science writer based in Santa Cruz.

Distributed analysis system

► biodas.org

Interoperable Informatics Infrastructure Consortium

► www.i3c.org

University of California, Santa Cruz, genome browser

► genome.ucsc.edu

Genome Knowledgebase

► www.genomeknowledge.org

Entrez system

► www.ncbi.nlm.nih.gov/Entrez

Ensembl genome browser

► www.ensembl.org

VISTA

► www-gsd.lbl.gov/vista

ROY KAUSCHMIDT/LBL

manual reference sets that you can rely on."

While Apweiler is tackling large-scale annotation, others are concentrating on finding genes and proteins linked to a particular process, such as a disease. The bioinformatics and drug-discovery company Inpharmatica in London, for example, provides annotation databases and tools to identify potential drug targets.

Because of the plethora of different names given to the same genes and proteins in different organisms, a growing trend is the use of 'ontologies' — controlled vocabularies in which descriptive terms (such as gene and protein names) and the relationships between them are consistently defined. One ontology that is now widely adopted is the Gene Ontology (GO), but it doesn't cover all biology, and others have developed their own, often complementary, ontologies. BioWisdom in Cambridge, UK, for example, sells information-retrieval and analysis tools for drug discovery based on proprietary ontologies in fields such as oncology and neuroscience.

Working as part of the Alliance for Cellular Signaling, a team led by Shankar Subramaniam is developing an ontology that captures the different states of a protein, such as phosphorylation state. This will serve as a foundation for the Molecule Pages, a literature-derived database of signalling molecules and their interactions.

GO coordinator Midori Harris at the EBI and her colleagues are encouraging developers of new ontologies to make them publicly available through GO's website. They hope this will not only drive standardization, but will help to expand GO's capabilities by allowing

the creation of combinatorial terms derived from different ontologies.

But most researchers agree that tools are only part of the solution. "The passion for biology often gets missed out here," says Birney. "People think it is all about finding technical solutions that magically solve problems, but frankly, far more important is really wanting to see the data hang together."

M.C.

Gene Ontology Consortium ► www.geneontology.org

European Bioinformatics Institute ► www.ebi.ac.uk

Alliance for Cellular Signaling ► www.afcs.org



Automated annotation: Ewan Birney and Ensembl.

HEIKKI LEHVASLAHO

Company	Products/activity	Location	URL
Sequence, genome and gene-expression analysis			
Accelrys	GCG Wisconsin package for sequence and genome analysis; Discovery Studio for database mining, genomics and proteomics	San Diego, California	www.accelrys.com
Affibody	Software for genomics data analysis and management	Bromma, Sweden	www.affibody.com
Aneda	Desktop bioinformatics tools for genomics and proteomics	Roslin, UK	www.anedabio.com
ApoCom Genomics	Desktop bioinformatics tools for gene prediction and gene-expression analysis	Knoxville, Tennessee	www.apocom.com
Array Genetics	Protein information database; tools for genomics and proteomics	Newtown, Connecticut	www.arraygenetics.com
BIOBASE	TRANSFAC family of databases; analysis tools for gene expression, promoters and signalling pathways; contract bioinformatics	Wolfenbüttel, Germany	www.biobase.com
Biocomputing	Data-management systems for genotyping and phenotype data	Espoo, Finland	www.biocomputing.fi
Bioinformatics Solutions	Desktop bioinformatics tools for sequence analysis and structure prediction	Waterloo, Canada	www.bioinformaticsolutions.com
BioTools	Analysis software for gene and protein sequences and chromatograms	Edmonton, Canada	www.biotools.com
Cognia	Bioinformatics software, including BIOBASE software and databases	New York, New York	www.cognia.com
Curagen	GeneScape portal for genome analysis tools	Branford, Connecticut	www.curagen.com
Digital Gene Technologies	TOGA gene-expression analysis software	La Jolla, California	www.dgt.com
DNASTAR	Desktop sequence-analysis and genome visualization software	Madison, Wisconsin	www.dnastar.com
Entigen	BioNavigator platform for sequence and genome analysis	Sunnyvale, California	www.entigen.com
GATC Biotech	Accelrys, DNASTAR and other bioinformatics software; DNA sequencing	Constance, Germany	www.gatc-biotech.com
Gene Codes	Sequencher sequence assembly and analysis software	Ann Arbor, Michigan	www.genecodes.com
Gene-IT	Universal software for database management and genomics	Le Chesnay, France	www.gene-it.com
Genomatix	Genome and sequence analysis tools; portals to mouse and human genomes	Munich, Germany	www.genomatix.de
Genomic Solutions	Proteomics bioinformatics tools	Ann Arbor, Michigan	www.genomicsolutions.com
Geospiza	Servers and tools for sequence assembly and analysis	Seattle, Washington	www.geospiza.com
Hitachi Software Engineering	DNASIS desktop bioinformatics software for DNA sequence assembly and analysis, and analysis of microarray data	Yokohama, Japan	www.hitachi-sk.co.jp/English/index.html
Inpharmatica	Biopendium and CeleraEdition Biopendium proteome annotation resources; PharmaCarta large-scale discovery informatics platform	London, UK	www.inpharmatica.com
InformMax	Vector bioinformatics software for sequence, genome and microarray data; Vector NTI for Macintosh; LabShare for data storage and management	Bethesda, Maryland	www.informaxinc.com
Iobion Informatics	GeneTraffic microarray data-management and analysis software	La Jolla, California	www.iobion.com
iSenselt	Microarray data analysis and storage software; oligonucleotide computation	Bremen, Germany	www.isenseit.com
LabBook	eLabBook web-enabled electronic notebooks; annotated human genome database and data-mining tools	McClean, Virginia	www.labbook.com
LabVelocity	Jellyfish desktop bioinformatics software; information services	San Francisco, California	www.labvelocity.com
LION Bioscience	Bioinformatics software, database development and management; DiscoveryCenter platform for data integration; contract bioinformatics	Heidelberg, Germany	www.lionbioscience.com
MiraiBio	DNASIS desktop software for DNA sequence assembly and analysis, protein sequence analysis, and analysis of microarray data	Alameda, California	www.miraibio.com
Molecular Biology Insights	Oligonucleotide identification software	Cascade, Colorado	www.oligo.net
Paracel	Software for sequence assembly, analysis and sequence-based genotyping	Pasadena, California	www.paracel.com
Premier Biosoft	Desktop bioinformatics packages for sequence analysis, primer design, and two-hybrid protein interactions	Palo Alto, California	www.premierbiosoft.com
PubGene	PubGene public access and commercial gene databases and analysis software	Oslo, Norway	www.pubgene.com
Redasoft	Genetic mapping and sequence analysis software and REBASE restriction enzyme database	Toronto, Ontario	www.redasoft.com
Rosetta BioSoftware	Rosetta Resolver gene-expression data analysis system	Kirkland, Washington	www.rii.com
Silicon Genetics	MetaMine, GeNet and GeneSpring microarray analysis software	Redwood City, California	www.sigenetics.com
science factory	BRENDA enzymology database; überTOOL bioinformatics platform for sequence, expression and structural data	Cologne, Germany	www.science-factory.com
Softberry	Software for sequence and genome analysis and database searching	Mount Kisco, New York	www.softberry.com
Southwest Parallel Software	Bioinformatics software packages	Albuquerque, New Mexico	www.spsoft.com
Textco	Desktop bioinformatics packages and electronic lab notebook	West Lebanon, New Hampshire	www.textco.com
X-MINE	Bioinformatics platform storage and analysis of genomics data	Brisbane, California	www.XMine.com
Databases			
Beilstein Information	Chemical databases	San Leandro, California	www.beilstein.com
Biomax Informatics	Annotated human genome database; customized data management	Martinsried, Germany	www.biomax.de
BioWisdom	Text search and pharmacology and oncology information databases	Cambridge, UK	www.biowisdom.com
Celera Genomics	Web-based tools for accessing the Celera annotated genomes databases; bioinformatics services	Rockville, Maryland	www.celera.com

Compugen	GenCarta annotated human genome, transcriptome and proteome database	Tel-Aviv, Israel	www.cgen.com
DECODON	Software for 2D-gel analysis and information storage	Greifswald, Germany	www.decodon.de
GeneLogic	Gene-expression databases and software for drug discovery	Gaithersburg, Maryland	www.GeneLogic.com
Iconix	DrugMatrix databases and software platform for chemogenomics research	Mountain View, California	www.iconixpharm.com
Incyte Genomics	Annotated gene and expressed sequence tag databases; Proteome BioKnowledge Library protein information databases; bioinformatics software	Palo Alto, California	www.incyte.com
Lexicon Genetics	Gene knockout and gene function databases and bioinformatics for drug discovery	The Woodlands, Texas	www.lexgen.com
LifeSpan BioSciences	Gene-expression and protein-localization databases and data-mining tools	Seattle, Washington	www.lsbio.com
MDL	Biological and chemical information databases; data-management software	San Leandro, California	www.mdli.com
Structural Bioinformatics	Protein and protein-structure databases; computational proteomics	San Diego, California	www.strubix.com

Computer systems, middleware and laboratory information management systems (LIMS)

Amersham Biosciences	Sierra Laboratory Workflow System for microarray and sequencing data	Piscataway, New Jersey	www.amershambiosciences.com
CLONDIAG	PARTISAN microarray LIMS	Jena, Germany	www.clondia.com
geneticXchange	K1 System middleware platform for biological data integration	Menlo Park, California	www.geneticxchange.com
Helixense	Software and system infrastructure supporting large-scale distributed computing and biological data management	Singapore	www.helixense.com
IBM	DiscoveryLink platform for database integration; data-management systems	White Plains, New York	www.ibm.com/solutions/lifesciences
Mitsui Knowledge Industry	LIMS; software for membrane protein secondary-structure prediction, data management and analysis of gene-expression and SNP data	Tokyo, Japan	bio.mki.co.jp
NEC	Computer systems and networks	Tokyo, Japan	www.nec-global.com
Proteodyne	LIMS middleware for integration of network-enabled laboratory software	Martinsried, Germany	www.proteodyne.com
Silicon Graphics	SGI servers for high-throughput computing, visualization and data management	San Francisco, California	www.sgi.com
Sun Microsystems	Servers and workstations for high-throughput computing; universal software platforms for networks	Santa Clara, California	www.sun.com
TimeLogic	DeCypher system for accelerated bioinformatics	Crystal Bay, Nevada	www.timelogic.com
TurboWorx	Open computational platforms for biological research data including bioinformatics	New Haven, Connecticut	www.turbogenomics.com

Services

Aber Genomic Computing	Design of data-mining and predictive modelling software	Aberystwyth, UK	www.abergc.com
AGOWA	Genome and expressed sequence tag analysis; automated sequence annotation customized bioinformatics services	Berlin, Germany	www.agowa.de
Bioinformatics Services	Computational biology; bioinformatics services	Rockville, Maryland	www.bioinformaticsservices.com
Chemical Computing Group	Bioinformatics software, services and computer-aided molecular design	Montreal, Quebec, Canada	www.chemcomp.com
Cyberell	Bioinformatics software and services	Helsinki, Finland	www.cyberell.com
ePitope Informatics	Epitope prediction over the web	Durham, UK	www.epitope-informatics.com
GeneData	Bioinformatics systems and services; database development and management	Basel, Switzerland	www.genedata.com
Genometrix	Genotyping, gene expression and bioinformatics services	The Woodlands, Texas	www.genometrix.com
Keygene	DNA fingerprint analysis software; contract genomics and bioinformatics services	Wageningen, The Netherlands	www.keygene.com
NuGenesis	Scientific data management services	Westborough, Massachusetts	www.nugenesis.com
Sagittus Solutions	Bioinformatics software development	Manchester, UK	www.sagittussolutions.co.uk
SRI International	Contract informatics services	Menlo Park, California	www.sri.com
Tripos	Chemical libraries; molecular modelling, pharmacophore perception and virtual screening software; contract informatics	St Louis, Missouri	www.tripos.com

General

ALMA Bioinformatica	Bioinformatics software, consultancy and training	Madrid, Spain	www.almabioinfo.com
Applied Maths	Gel fingerprint analysis and bioinformatics software; contract bioinformatics	Kortrijk, Belgium	www.applied-maths.com
Bio-Rad	WorksBase bioinformatics software for proteomics	Hercules, California	www.discover.bio-rad.com
BioSolveIt	Software for molecular modelling, small-molecule docking, protein threading; bioinformatics services and training	St Augustin, Germany	www.biosolveit.de
Dalicon	Bioinformatics software for large-scale data management and analysis	Nijmegen, The Netherlands	www.dalicon.com
MegaMetrics	Data-mining software for microarray, proteomics and SNP databases	Wyndmoor, Pennsylvania	www.megametrics.com
Molecular Mining	Data-mining software	Kingston, Ontario, Canada	www.molecularmining.com
Partek	Pattern recognition and interactive visualization software; consulting services	St Charles, Missouri	www.partek.com
Spotfire	DecisionSite analytical and statistical data-management software	Somerville, Massachusetts	www.spotfire.com
SPSS	Clementine statistical and data-mining software; Clementine microarray application template	Chicago, Illinois	www.spss.com
Zeptosens	SensiChip microarray systems	Witterswil, Switzerland	www.zeptosens.com

● See advertisement

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A youthful field

Switching from academia to industry automatically ensures a higher wage, right? Not according to a recent survey conducted by *Nature Biotechnology*. The journal's online survey of readers across academia and industry in Europe and the United States revealed lower-than-anticipated salaries, and a younger-than-expected average age. The 1,700 or so respondents reported an average annual salary of under \$50,000 — and about half of them were 34 years old or less.

Deeper analysis of the data reveals a gulf between two broad groups of scientists. The larger set is younger, paid less and more mobile within the job market. Members of the smaller group, on the other hand, are older, better paid and are more likely to split their time between multiple sectors.

It is likely that this gulf exists not just in industry, but in academia as well, according to similar studies published this year by the US National Science Foundation and the American Association for the Advancement of Science. Both of those studies revealed a higher average salary than was found by *Nature Biotechnology's* survey — but they also included a higher percentage of more senior scientists.

The results of all three studies illustrate a major problem with salary surveys — it is difficult to get a definitive average for a given field. And breaking the results down by age gives a less reliable total because of the inevitable sampling problems.

But even with such caveats, one can still draw some useful conclusions. Young scientists do not seem to be in it for the money — at least not immediately. Perhaps the lure of tenure or the uncertain promise of stock options keeps them striving. But maybe slightly higher salaries would be a better way to attract, and then sustain, young scientists, regardless of sector.

Paul Smaglik
Naturejobs editor



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Does travel broaden the scientific mind?

Rosemary Clyne:

Learn about the customs of your host country and if a foreign language is spoken, learn as much as you can prior to arrival.



When it comes to young scientific talent, there is a trade deficit between the United States and the rest of the world. Every year, hundreds of foreign-born postdocs take up positions in the United States, whereas only a fraction of that number of US-born scientists take up similar positions abroad.

Why the imbalance? Many US graduates fear that a stint abroad will jeopardize their chances at home by taking them out of the US science networking loop. But a growing minority believe that the experience broadens their horizons and increases their employment opportunities.

"I decided to go abroad for both professional and personal reasons," says Daniel Zucker, an astronomer at the Max Planck Institute in Heidelberg. He hasn't regretted the decision. Zucker, who studies the histories of nearby galaxies, says that the chance to do good science while experiencing another culture and learning another language attracted him to the US National Science Foundation's International Research Fellowship Program, which funds his Heidelberg post.

JOB-HUNTING FROM A DISTANCE

E-mail and the Internet may have ended the perception that working overseas leaves postdocs out of touch. But do stay in contact with former collaborators and advisers, warns Bianca Sclavi, a postdoctoral researcher at the Laboratory for Biotechnology and Applied Pharmacology at the Ecole Normale Supérieure de Cachan in Paris. "It's a good idea to go back for meetings to keep in touch, at least once a year," she says.

"The main disadvantage of working abroad is that I feel a bit out of the American job-search loop," says Darren Irwin, a guest researcher at the Molecular Population Biology Laboratory at Lund University in Sweden. "Travelling from Sweden to visit American universities is both time-consuming and expensive."

But this is a temporary disadvantage, says Irwin. Once back in the United States he suspects that his international experience will strengthen his chances of gaining a professorship. "It can be an excellent learning experience to see how scientists in other countries do their work and what subjects they are interested in," he says. "I feel that my knowledge has broadened

substantially as a result of working in both countries."

Psychologist Casey Cromwell did a postdoc at the University of Fribourg's Institute of Physiology in Switzerland from 1996 to 2000. After that he worked at Wake Forest University Medical School in Winston-Salem, North Carolina, before taking his current post at Bowling Green State University in Ohio. Cromwell says he never really felt out of the loop while abroad. "I was brought over to do a few interviews while I was still in Switzerland," he says. "I got telephone interviews from the States." The web and e-mail made it easy to find a job, he says, although he admits to being a bit worried before he landed the Wake Forest position.

PLUSES AND MINUSES

Making the move to another culture and language can seem daunting. But many of the people postdocs encounter, especially scientists, speak excellent-to-functional English. Postdocs often take a language course, sometimes paid for by their host lab. The main difficulties in adjusting have to do with the details of day-to-day life such as shopping for food, setting up a bank account and renting somewhere to live, all of which are done differently and in some cases take much longer than in the United States.

Rosemary Clyne, a postdoctoral fellow at Austria's Research Institute of Molecular Pathology in Vienna, counsels postdocs to be flexible and open-minded. "Austria's not the land of convenience that the United States is, but in my opinion this is part of its character," she says. But the trade-offs are worth it. "The quality of the institute's science and facilities far outweigh minor inconveniences like being unable to shop on Sunday," says Clyne. And Vienna has much to offer. "The cultural opportunities here rival any US city and a postdoc can afford to enjoy them," adds Clyne, who studies meiosis in the yeast *Saccharomyces cerevisiae*.

Her advice is to research the lab, institute and city as extensively as possible. "Choose a lab that is well-recognized and whose group leader has broad contacts. Learn about the customs of your host country and if a foreign language is spoken, learn as much as you can prior to arrival." And, she adds, be prepared for numerous visits from envious friends and relatives. ■

Karen Kreeger is a freelance science writer based in Philadelphia.

Web links

Research Institute of Molecular Pathology
 ♦ www.imp.univie.ac.at
 NSF International Research Fellowship Program
 ♦ www.nsf.gov/cgi-bin/getpub?nsf002149

Transitions

X Mary Lopez is to join PerkinElmer Life Sciences as director of biochemistry from Proteome Systems, where she served as executive vice-president of proteomics R&D.

X Biotech company Oxford BioMedica last month named **Nick Woolf** as senior vice-president of corporate strategy. Woolf was most recently director and head of European biotechnology research at ABN Amro.

X Affymetrix recently appointed Applied Biosystems' **Steve Lombardi** as vice-president of corporate development. The former senior vice-president of applications and products will be involved in early-stage product development.

X Last month **Alan Guttmacher** was named deputy director of the US National Human Genome Research Institute in Bethesda, Maryland, of which he has been a member since 1999.

X **Augustus Oemler** will step down as director of the Carnegie Observatories in 2003. No replacement has yet been named.

X **Paul Goldsmith** of Cornell University will step down as director of the National Astronomy and Ionosphere Center at the end of this year.

X In December, **Paul Kulesa**, a postdoc at the California Institute of Technology, will join the Stowers Institute for Medical Research in Kansas City, Missouri, as director of imaging technology.

X **Jaap Goudsmit**, co-founder of the International AIDS Vaccine Initiative and EuroVac, the European AIDS vaccine initiative, has succeeded **Ton Logtenberg** as chief scientific officer at biotech firm Crucell, in Leiden, the Netherlands.

PHARMACEUTICALS

Viagra's co-inventor **David Brown**, former global head of discovery at Roche Pharma in Basel, Switzerland, was last month appointed president and chief executive of Cellzome, a Heidelberg-based drug-discovery company. Brown, who has worked for four of the ten largest pharmaceutical companies in his 28-year career, became intrigued by Cellzome when researchers at the company, together with co-workers at the European Molecular Biology Laboratory in Heidelberg, elucidated the protein interactions of yeast (A.-C. Gavin *et al.* *Nature* **415**, 141–147; 2002).

"It hadn't occurred to me that it was possible to do proteomics on that scale," Brown says. Before that paper was published, he had lectured frequently on why several drug candidates were failing and had concluded that the reason wasn't the number of targets but the shortage of "chemically tractable" ones. Many targets weren't well validated; in other cases, compounds that bound to the targets proved to be toxic to animals or humans. After the paper appeared, he thought: "Maybe these guys have got something that will affect one or two of these issues." So he visited the company, was impressed with its proteomics technology platform and was convinced that the firm could use its work in yeast to elucidate biochemical pathways in humans.

He aims to expand Cellzome's drug-discovery capacity and use its technology to find new roles for "fallen angels" — compounds that were shelved after they didn't work for one disease, although they may still hold promise against another. Brown succeeds **Charles Cohen**, who will now serve as chairman of the company's supervisory board.

PROTEOMICS

David Muddiman has recently moved from the chemistry department at Virginia Commonwealth University to the Mayo Clinic in Rochester, Minnesota, to take up a position as professor of biochemistry and molecular biology. He also becomes director of the Mass Spectrometry Group for the Study of Biological Complexity, a division of the newly formed Mayo Clinic Proteomics Research Center.

Muddiman's work focuses on using mass spectrometry to address biological questions that have clinical impacts. He was attracted to the Mayo Clinic, he says, for the access it offers to clinical populations and the opportunity to carry out technology development.

Muddiman might not have taken up the post if he had listened to colleagues who,



David Brown



Bruce Runnegar



Sophie Combe



David Muddiman

because of his interest in basic science, warned him that this wasn't the right move. But he is convinced that the facility, which has nine mass spectrometers, will be conducive to both basic and clinical work and will help to foster collaborations with nearby institutes such as the University of Minnesota and the University of Wisconsin–Madison. "Ultimately, we'd like to make this a national resource," Muddiman says.

ASTROBIOLOGY

Australian palaeontologist **Bruce Runnegar**, of the University of California, Los Angeles, is to succeed Nobel laureate **Baruch Blumberg** as director of the NASA Astrobiology Institute. For the past four years, Runnegar has directed the Center for Astrobiology at the Institute of Geophysics and Planetary Physics, one of the Astrobiology Institute's 11 original lead teams. Educated in Australia at the University of Queensland, Runnegar became a Fellow of the Australian Academy of Science in 1987.

The NASA consortium's aim is to examine how life began and evolved, and whether life exists on other planets. "The answers to these questions will not come quickly," says Runnegar. "That's why NASA needs to attract bright young people to the field of astrobiology." Part of his role will be to develop educational opportunities to help meet that need.

DRUG DISCOVERY

Trigen, a London-based drug-discovery company, has been attracting managers from large pharmaceutical companies to complete its management team, bringing the number to four over the past six months. **Suresh Chahwala**, who managed cardiovascular drug discovery for Pfizer's site in Sandwich, Kent, and **Sophie Combe**, of Aventis in Paris, were appointed in August. They follow **Tony Kennedy**, former global head of project management with Roche in Basel, Switzerland, and **Oliver Boucher**, former GlaxoSmithKline director of business development, who joined in April. The growth in management follows the company's acquisition of the nonprofit Thrombosis Research Institute (TRI), spun out of King's College London.

Sanjay Kakkar, Trigen's chief executive, expects the 20-person company to almost triple in size by the end of 2003. The company may also consolidate into one location; at present the management office in central London is a short distance from the research site at the old TRI in Chelsea.

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